*Bulgarian Journal of Veterinary Medicine*, 2023 ONLINE FIRST
ISSN 1311-1477; DOI: 10.15547/bjvm.2023-0073

*Original article*

# SEPARATION AND PURIFICATION OF CHICKEN IgY WITH ITS FIELD EFFICACY IN CONTROLLING AVIAN INFLUENZA IN MUSCOVY DUCKS

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**Summary**

Radwan, F. M., A. A. El-Shemy, N. I. Toaleb, M. A. Bastamy & M. M. Amer, 2023. Separation and purification of chicken IgY with its field efficacy in controlling avian influenza in Muscovy ducks. *Bulg. J. Vet. Med.* (online first).

The study aimed to prepare pure immunoglobulin Y (IgY) and investigate its potential of use in avian influenza (AI) H5N1 control in naturally infected ducks. The IgY was prepared using the polymer precipitation method and was found to have high hemagglutination inhibition (HI) mean titres against H5, H9, and ND by the 5$^{th}$ and 8$^{th}$ week post-vaccination (wpv). The separated IgY had mean HI titres against H5 7.20±0.46 and 7.60±0.74 for 5 and 8 wpv, respectively. SDS-PAGE analysis showed the presence of two prominent bands with molecular weight 65.08 and 27.96 kDa at 5 wpv, which resolved into 65.98 and 27.96 kDa at 8 wpv. The protein concentration of IgY was 3.92 and 5.71 mg/mL at 5 and 8 wpv, respectively. The therapeutic potential of IgY was evaluated in 33-day-old Muscovy male ducks showing signs of AI H5N1 infection with 35% mortality within 36 hours. The ducks were injected intramuscularly (i.m.) with 1 mL IgY diluted 1/5 or 1/10 for three successive days. The results showed that the ducks injected with IgY had reduced mortality rates compared to the non-injected ducks. After the third injection, the ducks appeared to be normal, and the total mortality rate was 23.7% and 45.18% in 1/5 and 1/10 injected birds, respectively, compared to 98.08% in non-injected birds. In conclusion, the study suggests that IgY prepared from immunised hens can be used to control AI H5N1 in ducks. However, further investigations are needed to determine the accurate dose, time, and concentration of protein required for effective treatment.

**Key words**: AI-H5N1, chicken IgY, HI test, Muscovy ducks, RT-PCR, SDS-PAGE

## INTRODUCTION

Avian influenza virus (AIV) is a type A virus belonging to the family *Orthomyxoviridae*, pleomorphic, enveloped, with 8-segmented RNA, 80–120 nm of size (Spackman, 2020; Lamb & Krug, 2001). HPAI viruses (H5) or (H7) are usually

incriminated in severe, systemic disease with high mortality in chickens and other gallinaceous poultry (Pantin-Jackwood *et al.,* 2016; Akanbi *et al*., 2020). Usually, domestic ducks act as reservoir to many AIV subtypes and their reassortments, playing a role in emergence of new AIV genotypes and virus pathogenicity (Samir *et al*., 2018; Parvin *et al.,* 2020). AI viruses are still of major importance to global health due to their ability to undergo change through antigenic drift and antigenic shift (Kim *et al*, 2018; Ciminski & Schwemmle, 2021; Maleki *et al.,* 2021).

New HPAI H5N1 mutants started to induce severe signs in domestic ducks (Samir *et al.,* 2018). The HPAIV subtype H5N1 originating in the Chinese (GS/GD96) goose/Guangdong clade 2.2.1 was reported to be firstly introduced into Egypt in 2006 (Aly *et al.,* 2008; Abdelwhab & Hafez, 2011). Pantin-Jackwood *et al.* (2013) reported that Muscovy ducks (*Cairina moschata*) are susceptible to HPAI virus H5N1 infection by different routes accompanied by more severe clinical signs and higher mortality. Domestic ducks i.n. inoculated with H5N1 HPAI viruses frequently showed corneal opacity more than neurologic signs and mortality (Yamamoto *et al.,* 2015). Pathological lesions of HPAIVs infection in domestic ducks are various according to the geographical factors, while disease severity depends on the infected host, virus pathogenicity and virulence, and the associated secondary infections (Swayne *et al.,* 2013). Pancreatic haemorrhages with necrosis are frequently seen (Lean *et al*., 2022; Djurdjevic *et al.,* 2023).

IgY helps in fighting infectious disease in humans e.g. Covid 19 (Lee *et al.,* 2021; Ivanova *et al.,* 2022; Agurto-Arteaga *et al.,* 2022), in poultry including AI (Abbas *et al.,* 2019) and antibiotic resistance (Kollberg, 2015). It was postulated that avian IgY antibodies represent a low-cost, highly effective, and well-tolerated approach that can be scaled to produce enormous quantities of protective antibodies (Wallach *et al.,* 2011; Spillner *et al.,* 2012; Abbas *et al.*, 2019). The IgY can be administered passively in poultry orally and intranasally (i.n.), intramuscularly (i.m.) or subcutaneously (s.c.) (Pereira *et al*., 2019). In *in vitro* assays IgY was able to inhibit virus haemagglutination of the homologous and in some cases heterologous clades (Wallach, 2011; Du *et al.,* 2013; Nguyen *et al.,* 2017). The high IgY titres against AIV subtypes H5N1 and H9N2 in eggs, and their roles in neutralisation of both viruses was reported (Wallach *et al.,* 2011; Nahla *et al.,* 2018). IgY administered i.n. 1 h prior to H5N1 infection was 100% protective for mice against lethal challenge (Wallach *et al.,* 2011).

Pandemic influenza is still a serious threat to global health and the world economy. Passive immunisation could offer an alternative strategy to prevent and treat influenza virus infection (Nguyen *et al,* 2010). Chicken IgY has been used mainly for treatment of infectious diseases of the gastrointestinal tract. IgY from egg yolk from laying hens immunised with inactivated AIV (H5.H9) could provide protection against infections with HPAIV H5N1(Nguyen et *al,* 2010; Wallach *et al*., 2011). The IgY antibodies can be used as an effective means of immune prophylaxis for the prevention and control of both seasonal and pandemic influenza and can cross-protect against influenza viruses of different clades and strains (Nguyen, 2010; Wallach *et al.,* 2011; Ye *et al.,* 2012). The advantages of IgY are its easy preparation and low production costs (Karlsson *et al.,* 2004).

IgY has a number of potential applications in biotechnology and medicine, including use as an alternative to mammalian antibodies for diagnostic and therapeutic purposes. Muscovy ducks are known to be susceptible to AIV H5N1 and can serve as a useful animal model for studying the pathogenesis of the virus and evaluating potential treatments or interventions. Therefore, the primary objective of the study was to prepare egg yolk immunoglobulin Y (IgY) from eggs from vaccinated hens, assess its ability to neutralise the AI-H5N1 antigen, measure the protein concentration of the purified IgY as well as evaluate its effectiveness in controlling AI-H5N1 in naturally infected male Muscovy ducks.

## MATERIALS AND METHODS

### *Ethics approval*

The study was carried out according to guidelines for animal welfare and was approved for inclusion of the hens and ducks by the Institutional Animal Care and Use Committee, Vet. Cu. Iacuc. 2009: 2022525.

### *Extraction and preparation of IgY*

*Immunisation of hens:* One thousand red laying hens at 130 days of age were i.m. immunised with 0.5 mL of the inactivated AI (reassortant AIV H5 subtype) vaccine (inactivated strain Re -6+ strain Re -8+), ND (La Sota strain) and AIV H9 subtype vaccine produced by Zhaoqing Dahuanong Biology Medicine, China. Hen immunisation was performed 3 consecutive times, at 3-week intervals.

*Blood samples for serum.* Twenty clotted blood samples were collected from the wing vein for serum from layer hens at time 0 and 3 weeks after each booster immunisation to monitor HI antibodies titre against H5N1, H9 and ND antigens. Sera were heat treated at 56 °C for 30 minutes before being tested in HI assay (Sun *et al.,* 2018**)** (Table 1).

*Collection of eggs from immunised hens:* One hundred and fifty fresh clean eggs were collected, at 5 weeks and at 8 weeks from the last vaccination to extract IgY 1 and 2, respectively. The eggs were fumigated in a cabinet using a mixture of 40 mL formalin (40%) and 20 g of potassium permanganate/$m^3$, at temperature 24 °C and relative humidity between 60 to 80%, respectively, for 30 minutes (Olsen et al., 2017). The collected eggs were transported and kept under 4 °C for extraction of IgY.

**Table 1.** Result of HI titres against H5 in serum of vaccinated layer (mean ± SD; n=20)

| Vaccination | Week post-vaccination | Mean ±SD |
|---|---|---|
| 1st | 3 | 5.10± 0.73 |
| 2nd | 3 | 6.62± 1.3 |
| 3rd | 3 | 7.25± 1.63 |

### *Separation and extraction of IgY*

For extraction of chicken antibodies from egg yolk (Polson *et al*., 1980), the following materials were supplied: polyethylene glycol 6000, PBS, glass funnel, glass stick, falcone tube 50 cm and freshly collected clean eggs from immunised hens.

*Extraction of chicken IgY by polyethylene glycol (PEG) precipitation.* The high polymer precipitation to isolate IgY as described by Polson *et al*. (1980) was adopted. The purity of extracted IgY was measured by 10% SDS-PAGE (Fig. 1). The reactivity to H5N1, H9N2 and NDV were determined by HI test (Table 2).

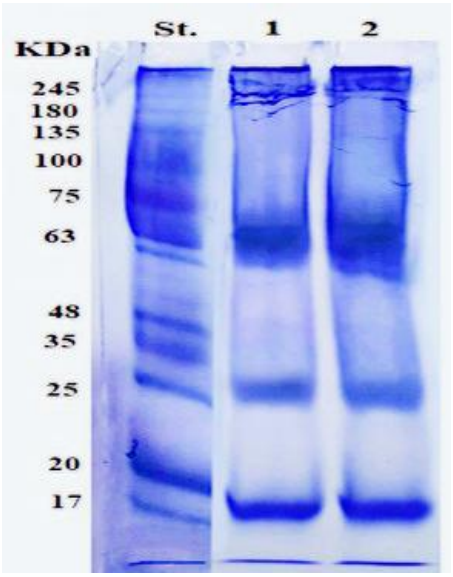


**Fig. 1.** Electrophoresis 10% SDS-PAGE profile of purified chicken IgY antibodies. The two IgY chains appeared resolving SDS-PAGE gel; lane St: weight marker standard ladder; lane 1: purified IgY from eggs 5 weeks after 3rd vaccination; lane 2: purified chicken lane of egg yolk 8 weeks after 3rd vaccination.

IgY 1 and 2 were stored in 5 mL sterile vials, transported, and stored at 4 °C till used for IgY separation.

*Evaluation of IgY*

*Haemagglutination inhibition (HI).* The inhibition of haemagglutination activity of the serum antibodies titre and separated IgY, as well as diluted 1/5 and 1/10, were determined by HI test lyophilised H9N2 and H5N1 antigens as well as La Sota NDV strain (Nguyen *et al.,* 2010; Abdel Rhman *et al.,* 2013). A 0.5% suspension of chicken red blood cells (RBCs) in PBS was prepared and stored at 4 °C until needed. The antigen was prepared in PBS to a concentration of 4 HAU/0.05 mL. A series of two-fold dilutions of the serum or IgY sample in PBS was prepared, starting from a concentration of 1:10. The diluted IgY was mixed with the NDV antigen and incubated at room temperature for 30 min. Each well of a U-shaped microtitre plate was then filled with 0.05 mL of the chicken RBC suspension, followed by the addition of 0.025 mL of the IgY-antigen mixture to the first well and subsequent transfer of 0.025 mL from the first well to the second well, until the final well. The plate was then incubated at room temperature for 30 min. The end-point titre was the highest dilution of the IgY that completely inhibited HA and was expressed as the reciprocal of the last dilution containing non-agglutinated RBCs. Positive laboratory serum was used as a control.

*Determination of IgY antibodies by electrophoresis (SDS-PAGE).* The following reagents were used: acrylamide 30% stock solution, separation buffer, stacking buffer, ammonium persulfate (APS): 400 mg/mL (stored at –4 °C in small aliquots), TEMED (NNN'N' tetramethyl ethylene diamine), sample buffer (2×) pH to 6.8, stored at –4 °C, and stock electrophoresis buffer (10×x). The protein content of

**Table 2.** Result of HI titres in egg yolk collected for IgY (mean ± SD; n=8)

| Vaccination | Week after the 3rd vaccination | H5 | H9 | ND |
|---|---|---|---|---|
| 3 times 3 weeks in-between | 5 | 7.87 ± 0.83 | 7.00 ± 0.75 | 7.20 ±0.46 |
| | 8 | 8.12 ± 0.98 | 7.12 ± 0.83 | 7.60 ±0.74 |

separated chicken IgY antibodies was performed in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli**,** 1970; Selvan *et al*., 2012) to determine the molecular weight of the purified chicken IgY antibodies. Chicken IgY was mixed with a sample buffer containing 2-mercaptoethanol before loading to the gel. After separation, the slab gel was stained with Coomassie Brilliant Blue dye. Relative molecular weights of bands were calculated using restrained molecular weights protein markers (Genedirex BL Ultra, USA) electrophoresed on the same gel. Molecular weights were determined using Bio-Rad Gel Doc XR+ (Fig. 1) apparatus (Sudjarwo *et al.*, 2017).

*Determination of protein in extracted IgY*

The procedure of Lowry (Waterborg & Matthews, 1984) is a widely used method for quantifying protein concentrations in biological samples. The assay works by measuring the colorimetric reaction of cuprous ions with peptide bonds in proteins under alkaline conditions. The intensity of the resulting blue colour is proportional to the amount of protein present in the sample. The following reagents were used: A) 10% $Na_2CO_3$ in 0.5N NaOH (0.2 g NaOH +1 g $Na_2CO_3$ in 10 mL); B) 0.5 % $CuSO_4$ in 1% Na-K tartrate; C) composed of 1 mL of Buffer B + 10 mL of buffer A; D) composed of 1 mL Folin reagent + 10 mL distilled water. The assay was performed as 0.1 mL of the IgY sample was added to 0.5 mL of buffer C and 0.4 mL of water, and the mixture was incubated at room temperature for 10 min. Then 2 mL of diluted Folin-Ciocalteu reagent was added to each tube, and the tubes were incubated at 50 $^{o}$C for 15 min. After cooling the tubes, the absorbance was measured at 720 nm using a spectrophotometer. The protein concentration of the IgY sample was determined by comparing its absorbance to a standard curve generated using known concentrations of a protein standard.

*Field application of IgY*

*Duck flocks history and sampling.* Muscovy ducks flock contained 900 male birds (33 days of age from a replacement herd for semen for artificial insemination reared on wheat straw) showing clinical signs including off-food nervous signs (torticollis), respiratory signs, recumbency, incoordination of movement, corneal opacity, and increased mortality (Fig. 2 and 3). Mortality reached 35% (315/900)

**Fig. 2.** Nervous signs in a Muscovy duck

**Fig. 3.** High mortality

in 36 hours from start of clinical signs. After field investigation and collection of flock history, diseased ducks were transported hygienically into our clinic room for clinical and postmortem examination, and collection of samples for laboratory examination. Postmortem lesions were congestion of the liver, spleen, and lungs that appear after more than 48 hours from clinical signs. Pancreatic haemorrhage with necrosis was detected (Fig. 4). The liver, spleen, and pancreas were collected for the detection of H5N1 RNA by RT-PCR.

*AI H5N1 diagnosis by one-step RT-PCR.* Methods of Slomka *et al*. (2007) were used. The viral RNA was extracted from homogenised pooled tissues samples/ bird using QIA amp viral RNA extraction Kits according to the manufacture's protocol. One step RT- PCR was carried using QIAGEN® One Step RT-PCR kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RT-PCR was used for the detection H5N1 using the following primers: H5 Forward primer (25 bp) 5' ACATATGACT ACCCACARTATTCAG3', H5 Reverse primer (20 bp) 5'-AGACCAGCTAY CATGATTGC3', H5 prob 5'-Fam-TCWACAGTGGCGAGTTCCCTAGCA-BHQ3'. Four thermal cycles for RT-PCR were run: the 1st was 15 min at 55 °C, the 2nd one: polymerase activation 1 min at 95 °C, the 3rd one: 40 denaturation cycles for 10 sec at 95 °C and the last: annealing for 30 sec at 60 °C. The local isolate of AI H5N1 (A/chicken/Egypt/Q1089E/ 2010) with accession number KF881634.1 was used as positive control.

*Intramuscular administration of IgY.* Thirty-six hours from start of signs and AI confirmation by RT-PCR, IgY was administered passively to ducks with mild signs or apparent healthy birds to control infection and mortality. Duck groups were i.m. injected with 1 mL of 1/5 or 1/10 diluted IgY for 3 successive days in thigh muscles. Infected ducks were divided into 3 groups. Group 1 and 2 each had 135 ducks, submitted to three successive injections with 1/5 or 1/10 diluted IgY, while group 3 (260 birds) were kept as non-injected control. All groups were under daily observation for 10 days from the 1st injection.

## RESULTS

The results of HI test (Table 1) on sera collected 3 weeks after each vaccination dose showed that the obtained HI $\log_2$ titres against H5 were 5.1 ± 0.73 ; 6.62± 1.3 and 7.25± 1.633 3 weeks after 1st, 2nd and 3rd vaccination.

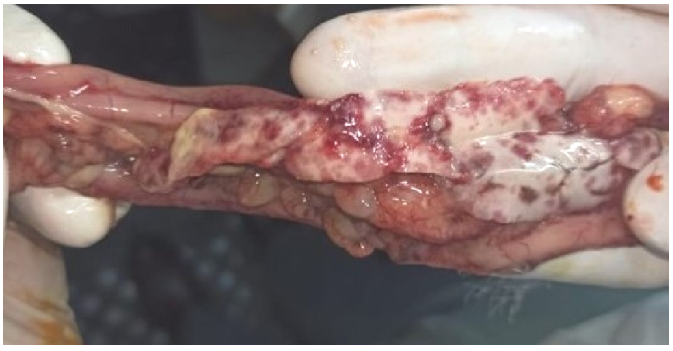

**Fig. 4.** Pancreatic haemorrhage in a male Muscovy duck.

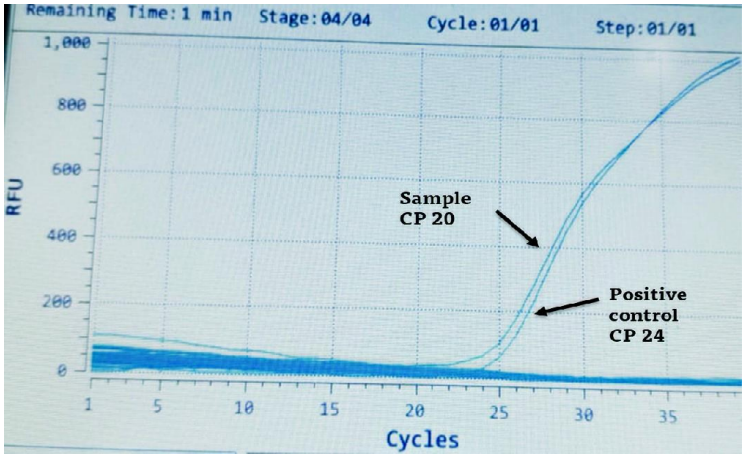


**Fig. 5.** Real-time PCR curve for H5 showing a positive result with CT 20.

Eggs for IgY were collected at 5 and 8 weeks post last vaccination. From each egg, about 1 mL/egg of IgY was extracted and purified. The separated IgY from each round was mixed and stored in 5 mL sterile ladled tubes at –20 $^{o}$C for further use. Eight tubes/time were tested for HI antibody titres (Table 2). The mean titres were 7.20 ± 0.46 and 7.60 ± 0.74 against H5, 7.00 ± 0.75 and 7.12 ± 0.83 against H9 as well as 7.87 ± 0.83 and 8.12 ± 0.98 against ND at 5 and 8 wpv, respectively.

Electrophoresis of purified chicken IgY antibodies in SDS-PAGE of samples from IgY separated at 5 and 8 wpv revealed three protein bands with molecular weights of 180.65 kDa, 69.75 kDa, and 27.52 kDa, respectively (Fig. 1) Consequently, the heavy IgY chain molecules weighing 65.08 and 65.98 kDa were detected at week 5 and 8 after the 3$^{rd}$ vaccination. The IgY light chain weighting 27.96 kDa was seen at week 5 and 8 after the 3$^{rd}$ vaccination by SDS-PAGE.

Protein concentrations of extracted purified IgY measured by Lowry assay by the 5$^{th}$ and 8$^{th}$ weeks after the third vaccination was 3.92 and 5.71 mg/mL, respectively. RT-PCR on extracted viral RNA against primers for detection of AIV H5 RNA give positive results at CT 20 (Fig. 5).

The used working solution was prepared by mixing equal volume of separated IgY from 5 and 8 wpv to have protein concentration of 4.85 mg/mL. HI titres of working dilution of IgY for injection of diseased ducks were 6.60± 0.74 and 5.6±0.69 for AI H5, 6.38 ± 1.30 and 5.50 ±1.19 for AI H 9 as well as 6.87±0.99 and 5.8±0.83 for ND in dilution 1/5 and 1/10, respectively (Table 3).

Injection of IgY antibodies resulted in several positive outcomes in ducks infected with H5N1 influenza virus. Specifically, 24 hours after the first injection, the injected ducks became more active, stopped dying, and increased their feed and water consumption. After the first injection, duck mortality decreased from 32.70% in non-injected ducks to 14.81% and 28.14% in ducks receiving 1 mL of 1/5 and 1/10 diluted IgY, respectively (Table 4). The day after the second injection, the injected ducks became even more active, showed milder clinical signs, and no additional birds became diseased. Furthermore, mortality was reduced to 6.67%

**Table 3.** HI titres against H5, H9 and ND in 1/5 and 1/10 diluted purified IgY for duck injection (mean ± SD; n=8).

| Dilution | H5 | H9 | ND |
|---|---|---|---|
| 1/5 | 6.60 ±0.74 | 6.38 ± 1.30 | 6.87 ±0.99 |
| 1/10 | 5.60 ±0.69 | 5.50 ±1.19 | 5.80 ±0.83 |

**Table 4.** Mortality in IgY-injected and non-injected naturally infected ducks

| Dilution | Day post 1st injection | Total number of birds | Mortality post injection | | Total | |
|---|---|---|---|---|---|---|
| | | | No of deaths | % | Mortality | % |
| 1/5 | 1 | 135 | 20 | 14.81 | | |
| | 2 | 105 | 7 | 6.67 | | |
| | 3 | 98 | 3 | 3.06 | | |
| | 4–10 | 95 | 2 | 2.11 | 32\135 | 23.70 |
| 1/10 | 1 | 135 | 38 | 28.14 | | |
| | 2 | 105 | 12 | 11.42 | | |
| | 3 | 93 | 8 | 8.60 | | |
| | 4–10 | 85 | 3 | 3.65 | 61\135 | 45.18 |
| Non-injected | 1 | 260 | 85 | 32.70 | | |
| | 2 | 175 | 65 | 37.71 | | |
| | 3 | 90 | 83 | 92.22 | | |
| | 4–10 | 7 | 2 | 28.57 | 255/260 | 98.08 |

and 11.42% in ducks receiving IgY diluted 1/5 and 1/10, respectively, compared to non-injected ducks (37.71%). After the third injection, the injected ducks restored their health and appeared normal, with mortality rates of 3.6%, 8.0%, and 92.22% in ducks receiving IgY diluted 1/5, 1/10, and non-injected ducks, respectively. By the end of the 10th day of observation (7th day after the last injection), the total mortality was 23.7%, 45.18%, and 98.08% in ducks receiving IgY diluted 1/5, 1/10, and non-injected ducks, respectively. Overall, these results suggest that the injection of IgY antibodies may be an effective method for reducing mortality and improving the health of ducks infected with H5N1 AIV.

## DISCUSSION

The approach of using specific IgY for prevention and therapy of influenza virus infection offers an alternative to current immunotherapy, which uses HPAIV H5N1 convalescent plasma (Kong *et al*., 2006), and an additional therapeutic option to antiviral drugs since widespread drug resistance has been recently reported among influenza virus strains. Current FDA-approved anti-influenza viral drugs consist of the adamantane compounds (amantidine/rimantidine) and the neuraminidase inhibitors oseltamivir and zanamivir (Gubareva *et al*., 2000). Average volume of egg yolk (15 mL) contains 50–100 mg of IgY, of which 2%–10% can

be of specific antibodies (Ferreira Júnior *et al*., 2012).

The molecular weight patterns agreed with the expected molecular mass for the heavy and light chains, respectively. The two bands with molecular weight 65.08, 27.96 kDa of egg yolk antibody IgY according to the protein ladder (Fig. 1, lane 1) were 65.98, 27.96 kDa of egg yolk antibody IgY (Fig. 1, lane 2). The results of the SDS-PAGE analysis revealed three protein bands with molecular weights of 180.65 kDa, 69.75 kDa, and 27.52 kDa, respectively, indicating the presence of whole IgY, Fc heavy chain, and Fab light chain (Selvan *et al*., 2012, Amro *et al*., 2018; Shimelis *et al.,* 2022).

The detected clinical signs and mortality in ducks were due to HPAI H5N1 subtype (Aly *et al.,* 2008; Abdelwhab & Hafez, 2011; Pantin-Jackwood *et al.,* 2013). Domestic ducks inoculated i.n. with H5N1 HPAI viruses showed corneal opacity more than neurologic signs and mortality (Yamamoto *et al.,* 2015). Pancreatic haemorrhages with necrosis lesions were seen (Lean *et al.,* 2022; Djurdjevič *et al.,* 2023*).* RT-PCR results with CP 20 with H5N1 primers indicated the specific detection of H5N1 AIV in field specimens (Ng *et al.,* 2006).

HI titres of working dilution of IgY for injection of diseased ducks were 6.60± 0.74 and 5.6±0.69 for AI H5, 6.38 ± 1.30 and 5.50 ±1.19 for AI H9 as well as 6.87±0.99, 5.8±0.83 for ND in dilution 1/5 and 1/10, respectively (Table 3). The serum HI antibody titre is often used as a surrogate marker of protection and is an important immunogenicity measure in chickens against ND (Shimelis *et al*., 2022), and AI (Ohmit *et al.,* 2011).

After the 3$^{rd}$ injection birds restored their health and become apparently normal. The mortality declined and by the end of the 10$^{th}$ day of observation (7$^{th}$ day after last injection) the total mortality was 23.7% (32/135), 45.18% (61/135) and 98.08% (255/260) in 1/5 IgY, 1/10 IgY and non-injected birds, respectively. The improved signs and decrease in mortality indicate that IgY was effective in control of AI H5 in ducks. This result can be supported with IgYs inhibiting the homologous as well as in some heterologous clades and strains of virus (Du *et al.,* 2013; Nguyen *et al.,* 2017), the presence of high IgY titres against AIV subtypes H5N1 and H9N2 in eggs, and the possible role of egg yolk IgY in neutralising the virus (Wallach *et al*., 2011; Du *et al*., 2013; Nguyen *et al*., 2017; Nahla *et al*., 2018).

The efficacy of IgY appears to be specific to viruses of the same HA type, as demonstrated by El-Kafrawy *et al.* (2021). For example, H5N1-specific IgY provided protection against infections with HPAIV H5N1 and H5N2 strains. The vaccine used to immunise the hens contained both H5 and H9 antigens, which may have contributed to the heterosubtypic immunity against influenza A viruses, including recently emerged avian H5 and H9 viruses, as reported by Sambhara *et al.* (2001) and Tamura *et al. (*2005). IgY has been shown to inhibit virus haemagglutination, both in homologous and heterologous clades and strains, as demonstrated by Du *et al.* (2013) and Nguyen *et al.* (2017). The HI test demonstrated that the HA activity of the virus was inhibited when tested against the homologous virus used for immunisation of hens (El-Kafrawy *et al.,* 2022). Nguyen *et al.* (2010) demonstrated that IgY antibodies against H5N1 isolated from eggs can protect against H5N1 and H5N2 viruses in mice.

The use of specific IgY antibodies as a therapeutic and prophylactic agent against H5N1 influenza virus infection in ducks

has several potential benefits, including high specificity, large-scale production, oral administration, low risk of adverse reactions, and potential for heterologous protection.

In conclusion, our *in vivo* results demonstrate the feasibility of using the IgY approach to produce large quantities of IgY for the potential prevention and control of lethal H5N1 infection when injected intramuscularly 36 hours after infection in ducks. The IgY specific to the immunised hens is easy to prepare and the injection of such IgY can help to control AI H5N1 in ducks. However, additional investigations are needed to determine the accurate dose, time, and protein concentration.

## ACKNOWLEDGEMENTS

The authors thank the Department of Poultry Diseases, Faculty Veterinary Medicine, Cairo University, Parasitology and animal disease Department, Veterinary Research Division, National Research Centre, Giza. Egypt, and Egyptian laboratory for poultry health, Bader Center, El-Behera for facilities during this study. This work was self-funded by team members and all authors declared that they did not receive any specific fund for this study.

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Paper received 12.06.2023; accepted for publication 04.08.2023

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