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CHARACTERIZATION OF QUAIL IGY PRODUCED AGAINST NEWCASTLE DISEASE VIRUS

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ABSTRACT

The IgY technology can be used for the production of antibodies for immunological analysis, disease diagnosis and control. Newcastle disease (ND) is one of the most important viral diseases affecting birds worldwide and is responsible for serious economic losses to the poultry industry due to high mortality, decreased egg production, and body weight loss. Further, it requires additional approaches together with vaccination to overcome its endemicity. In this study, a total of 50 Japanese quails (Coturnix coturnix japonica) 4-week old were used for the production of IgY against NDV genotype VII. The produced quail IgY was tested for titers and specificity using Hemagglutination-inhibition (HI) and agar gel immune diffusion test (AGID). The produced IgY was tested for purity by SDS-page analysis. HI showed gradual increase in the IgY titers till the end of the experiment. Further, AGID showed positive interaction between the produced IgY and filed samples infected with NDV. This collectively indicates the development of significant titers of purified IgY against NDV genotype VII virus in quail eggs.

KEYWORDS: Newcastle disease virus, Quails, IgY, HI, AGID.

1-INTRODUCTION

The avian egg contains all the important nutrients and growth factors required for the developing embryo including antibodies, which are transported from the blood of the hen into the egg yolk to provide immunity to the chick **(Yigani and Korver, 2010)**. Birds transmit maternal antibodies to their offspring by depositing the antibodies in the egg **(Brambell, 1970)**. There are three classes of antibodies in chickens, namely IgY (IgG), IgA, and IgM. In eggs, IgY is present mainly in the egg yolk **(Leslie and Clem, 1989)**, whereas IgA and IgM are present in the egg white due to mucosal secretion in the oviduct **(Rose *et al.*, 1974)**. Immunoglobulins IgY represents about 75% of the total immunoglobulins in poultry. Egg yolk contains over 100 mg of IgY per egg **(Criste et al., 2020)**.

Serum antibodies of hens are efficiently transferred and accumulated in egg yolk providing a valuable source of antibodies **(Gopel *et al.*, 2004)**. Thus, birds are used as an immunization host for egg yolk antibodies (IgY) production instead of IgG from mammals **(Nikbakht *et al.*, 2009)**.

Quails possess unique characteristics which make them a very good choice for producing hyper immune egg as fast growth, early sexual maturity (they lay their first egg at ~40 days of age), high rate of egg production (up to 250 eggs a year) and shorter egg incubation period (16-17 days) **(Priti and Satish, 2014)**. Additionally, due to their small body size (230- 250 g and 250-300 g for adult male and females, respectively), quails require less housing space and feed **(Padmakumar *et al.*, 2000)**.

Newcastle disease (ND) is one of the most important viral diseases affecting birds worldwide and is responsible for serious economic losses to the poultry industry due to high mortality, decreased egg production, and body weight loss **(Alexander, 1997)**. The causative agent, ND virus (NDV), belongs to genus Avulavirus of the Paramyxoviridae family. Virulent NDV continues to be endemic in many countries, despite the application of vaccines **(Dimitrov et al., 2016)**.

NDV infections are manifested through a wide range of strain-dependent symptoms including those within the respiratory system (coughing, sneezing, and wheezing), the nervous system (twisted neck, tremors, and paralysis), and the reproductive system (decreased egg production). Mortality rates may reach as high as 100% in unvaccinated flocks **(Ashraf and Shah, 2014)**. Unsurprisingly, NDV infections are responsible for

considerable economic losses to poultry production in both developed and developing countries **(Diel et al., 2012)**. In Egypt, recent outbreaks in poultry flocks were due to NDV that belongs to class II, genotype VII subgenotype VIIb and subgenotype VIId **(Moharam et al., 2019). Hezema et al., (2020)** demonstrated the ability of producing significant titers of IgY against NDV, however, in chicken.

In this study, we aimed to produce and characterize quail hyperimmune egg against NDV genotype VII with possible applicable values.

2-MATERIAL AND METHODS

2.1. Birds

A total of 50 4-week old Japanese quails (Coturnix coturnix japonica) were obtained from local breeding unit at Al yasmeen farm for quail production in Kafr El Sheik governorate. They were divided into 2 groups: forty injected with inactivated NDV genotype VII and ten as a control group. The birds were housed in animal facility joined with the Department of Microbiology, Faculty of Veterinary Medicine, Damanhur university as 10 birds per cage of 90 × 60 × 35 cm. The birds were raised in floor pens and given diet "*ad libitum*" based on a complete feed mix containing 21% total protein designed for adult birds at this stage with full access to water under a regimen of 14 hours of light and 10 hours of darkness at room temperature maintained at 27±2ºC **(Kassim., et al., 2011)**. The birds were left for 2 weeks for acclimatization.

2.2. Newcastle Disease virus

Local Egyptian Newcastle disease virus genotype VII (AOAV-1/Egy/Ch/R78/ 2018) with a titer of $10^{8.3}$ EID50/ml **(Reed and Muench, 1938)** was used. The haemagglutination titer was equal to log 2^7. The virus was kind gift from **Abd-elaziz, (2020)**. It was used in the production of specific IgY from quail after inactivation according to **King, (1991)**, in haemagglutination-inhibition test and in AGID.

2.3. Immunization schedules

The birds were divided into two groups; 40 bird were injected with inactivated NDV genotype VII and 10 birds were injected with sterile phosphate buffer saline (PBS) as a control. Birds were intramuscularly injected in the thigh muscle with an initial dose containing 0.25 ml of inactivated NDV genotype VII emulsified in an equal volume of Freund's complete adjuvant (Sigma -Aldrich-F5881) then subsequently followed by three booster doses. The booster doses were emulsified in incomplete Freund's adjuvant (Sigma -Aldrich-F5506) at a ratio of 1: 1 and injected I/M at 2, 4 and 6 weeks after initial immunization **(Zhen et al., 2008; Kassim., et al., 2011 and Najdi et al., 2016)**. Regular blood collection was performed every week from the wing vein throughout the experiment (8 weeks). The collected sera were stored at -20°C until used for further detection of specific IgY. Egg was collected daily for purification of IgY.

2.4. Extraction and purification of IgY

IgY was extracted and purified from egg yolks according to the method adopted by **Akita and Nakai, (1993)**. The eggs were opened, egg white was discarded and the yolk was collected, washed with PBS and stored at 20°C until purification **(Wooley and Landon, 1995 and Zhen *et al.*, 2008)**. Briefly, 5 g of egg yolk was 6 fold diluted with 10 mM phosphate buffer (pH 5- 5.2) and homogenized thoroughly using vortex. The sample mixture was centrifuged at 12,000xg for 30 minutes at 4°C to remove the lipid-rich precipitate. The supernatant, consisting of lipid-free fraction, was collected and precipitated with 40% ammonium sulfate (w/v). After centrifugation, the pellet containing the IgY enriched fraction was dissolved in 2ml PBS. To eliminate the residual salt, the purified IgY was dialyzed against PBS for 24 hours. The final IgY was stored at -20°C. The purity of purified IgY was checked using SDS-PAGE analysis according to the method adopted by **Gallagher, (2012)**.

The quantity of the purified IgY was measured by Bradford protein assay according to **He, F (2011)**. The purified IgY samples showed antibody concentrations of 11.16 µg/ 100ml.

Agar gel immunodiffusion test (AGID) and Hemagglutination-inhibition (HI) tests

AGID was carried out on the immunoglobulin Y samples using the methods described by **Jenson, (2014).**

The purified IgY was tested for the presence of anti-NDV IgY and specificity in quail egg yolk samples using AGID test and Hemagglutination-inhibition (HI) **(OIE, 2018b)**. The antibody titer in serum was measured by HI according to **OIE, (2018b)**.

Eleven field samples suspected of infection with NDV were collected from Elgarbia governorate. The collected field samples were processed according to **OIE, (2018b).** The samples were injected in embryonated chicken eggs and the allantoic fluid was collected then examined for the detection of NDV by HA **(OIE, 2018b)**. The positive HA samples (8 samples) were examined with chicken serum from chicken confirmed with PCR as Newcastle positive and H5N1 and H9N2 negative **(Abd-elaziz, 2020)**. The positive samples were used to assess the specificity of the purified IgY by AGID.

3. RESULTS

3.1. Purification and detection of egg yolk IgY

IgY against NDV genotype VII were purified according to **Akita and Nakai, (1993)**. The purified IgY fraction was subjected to SDS-PAGE analysis according to **Gallagher, (2012)**. The antibody showed two major bands with a molecular weight of ~25 KDa and ~68 KDa corresponding to light and heavy chains, respectively (Fig 1).

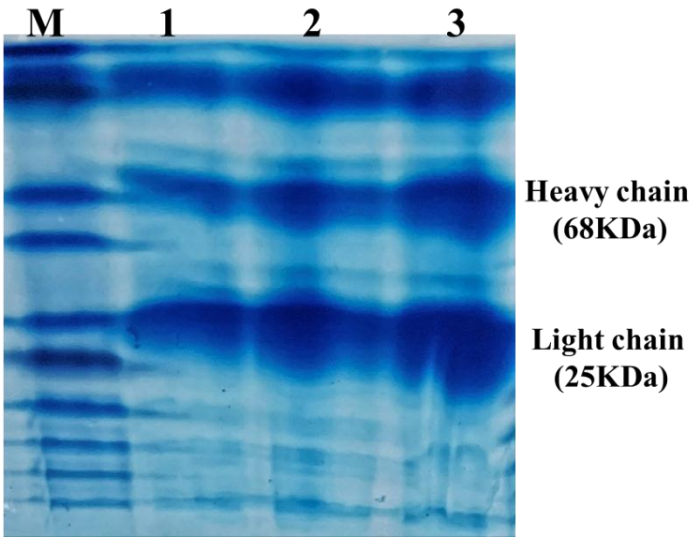

Figure (1): SDS-PAGE analysis of purified egg yolk IgY. M; standard molecular weight protein marker, 1, 2, 3; purified egg yolk IgY against NDV genotype VII.

3.2. Evaluation of antibody titers in serum and yolk of quail by HI test

HI test was employed to investigate the production of anti-NDV IgY in quail serum and measuring the titer of antibody along the experiment to determine the peak of anti -NDV IgY production in serum. Serum HI antibody titer values mean (GMT) against NDV genotype VII at 0 day of the experiment was 2^{0}. These values increased gradually to $2^{1.7,}$ 2^{4}, $2^{6.5}$, $2^{7.6}$, $2^{7.7}$,$2^{7.6}$, $2^{7.7}$ and $2^{7.5}$ in the 1st week, 2nd week, 3rd week, 4th week, 5th week, 6th week, 7th week and 8th week after initial immunization, respectively. Reaching the peak at the 4th week which was maintained over 5 weeks. There was no significant changes in IgY titers in the control group (anti-NDV IgY in serum equal to 0).

3.3. Agar gel immune diffusion test (AGID)

AGID test was employed to evaluate the specificity of the produced IgY to Newcastle disease virus and evaluate the possible role of the produced IgY in diagnosis of NDV in 11 field samples collected from Elgarbia governorate (Fig 2). The result showing precipitation line between IgY and control positive well and between IgY and sample well that revealed the presence of NDV in 5 from 11 collected field samples.

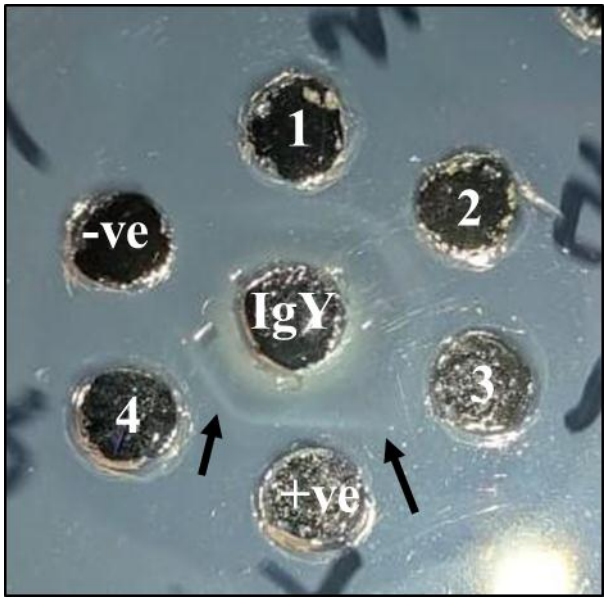

Figure (2): AGID for purified quail IgY against field samples (1,2,3,4) showing precipitation line (arrows) between IgY and control positive well (+ve) and between IgY and sample well, -ve; control negative.

4. DISCUSSION

The production of specific IgY against many different antigens has been studied by **Dubie et al., (2014)** as well as other large number of researchers and its application as an immunotherapeutic agent including its use for oral passive immunization against enteric pathogens has been

extensively reported. The IgY was highly effective in vitro both in the hemagglutination inhibition test and by serum neutralization of live influenza virus **(Wallach et al., 2011)**. IgY neither activate mammalian complement nor interact with mammalian Fc receptors that could mediate inflammatory responses in the gastrointestinal tract. Due to its distinctness from IgG, IgY has also been found to be advantageous in several techniques as well as in immune-affinity purification, in many cases replacing IgG **(Dubie et al., 2014)**. Further, the sustained antibody production in chicken is as well advantageous **(Dias da Silva and Tambourgi, 2010)**.

Quails are relatively sturdy birds that are resistant to several infectious diseases and environmental stresses. They are less expensive, easily manageable in terms of housing, and experimentally convenient due to their small size **(Scholtz et al., 2010)**. Quails require fewer volumes of antigens for immunization that is an advantage for producing antibodies against viruses and recombinant proteins that can be purified at low concentrations **(Somowiyarjo et al., 1990)**. They achieve sexual maturity at seven weeks of age as opposed to the 20–24 weeks required for a hen to start laying eggs. Moreover, quails have a high rate of egg laying (280–300 eggs per year), and immunization does not result in the reduction of egg production **(Najdi et al., 2016)**.

In the current study, quail IgY anti-Newcastle disease virus genotype VII were produced and characterized.

Efficient and simple purification techniques for immunoglobulin separation from egg yolk are necessary if birds are to gain general acceptance in antibody production. In this study, the IgY antibody was purified from egg yolks according to **Akita and Nakai, (1993)**. The purified IgY antibody titer was measured using HI test according to **OIE, (2018b)**. The results showed that antibody titer values mean (GMT) against NDV genotype VII at 0 day of the experiment was 2^0. These values increased gradually to equal $2^{1.7}$, 24, $2^{6.5,}$ $2^{7.6,}$ $2^{7.7}$,$2^{7.6}$, $2^{7.7}$ $2^{7.5}$ in 1st week, 2nd week, 3rd week, 4th week, 5th week, 6th week, 7th week, 8th week after immunization, respectively, reaching the peak at the 4th week that was maintained over 5 weeks. These results are supported by **Veguilla et al., (2011)** who reported that the HI assay was more specific for detecting antibodies against 2009 H1N1virus and also **by Rowe et al., (1999)** who showed that the HI assay is the standard method for serological detection of influenza virus infection in humans, and has been shown to be less sensitive for the detection of antibodies induced by avian influenza viruses. Also, this result agrees with **Sudjarwo et al., (2017)** who found that the IgY anti-MTBC (*Mycobacterium Tuberculosis*) concentration in egg yolk increased at 2 weeks and reached a maximum at 4 weeks after immunization.

The hyperimmune IgY purified from the eggs collected after reaching the peak in serum (5th week) had HI titer of 2^7, because the serum antibodies peaked one weak prior to the antibody peak in the yolk as mentioned by **Ling et al., (1998)** and **Abo-Ghanema et al., (2016)** who showed that the increase of antibodies in yolk began one week later than in serum.

The injection of the antigen by the intramuscular route results in higher antibody levels and higher specificity, being over 10 times more specific **(Wooley and Landon, 1995)**. On the other hand, extensive studies by **Schwarzkopf et al., (2000)** showed that the S/C antigen injection provoked a higher antibody titer than injection through the I/M route. In the intramuscular route, the immune response seems to work earlier than in the subcutaneous route, but eventually the subcutaneous route reaches the same level, and even higher **(Salomo. H., 2015)**.

The structure of IgY is significantly different from that of mammalian IgG despite the similarity in function **(Carlander et al., 1999)**. IgY contains two heavy (H) and two light (L) chains and has a molecular mass of 180 kDa, larger than that of mammalian IgG (159 kDa). IgY possesses a larger molecular weight H chain (68 kDa) as compared to that from mammals (50 kDa) and two light (L) chains with the molecular mass of 25 KDa each **(Warr et al., 1995)**. These structure differences are reflected in different molecular and biochemical interactions and may affect the electrophoretic mobility of IgY molecules as reported by **Michael et al., (2010)**. In this study, the purity of IgY was monitored by SDS-PAGE analysis (Fig. 1). When IgY was electrophoretically separated under reducing conditions, there was two major bands with a molecular weight of 25 KDa and 68 KDa that correspond to light and heavy chains, respectively. This finding is in agreement with those reported by **Kassim et al., (2011)** and **Esmailnejad et al., (2019)** who obtained two bands of light and heavy chains of quail IgYs at the same molecular weight. Unlike a study conducted by **Nasiri et al., (2016)** who showed that IgY contained two major proteins; 23 kDa (light chain) and 68 kDa (heavy chain). Also, **Zhen et al., (2008)** and **Abo-Ghanema et al., (2016)** showed two major bands with a molecular weight of 26 KDa and 65 KDa that correspond to light and heavy chains, respectively.

Maternal antibodies transfer can be defined as the passage of antibodies by mother to her offspring through colostrum or egg **(Grindstaff *et al.*, 2003)**. Birds transmit maternal antibodies to their offspring by depositing antibodies in their eggs **(Brambell, 1970)**. **Losch et al., (1986)** reported that laying hens transfer all serum antibody isotypes including IgG, IgM and IgA to their eggs. Further, two possible routes of transfer exist, one where antibodies in hen's serum are secreted into the maturing egg follicles and thus into the yolk. In the other route, antibodies in the oviduct are incorporated into the egg white along with the secreted albumin. They also reported that IgG transfer to ovarian follicles is receptor

dependent and the ovarian receptors allow selective transport of all IgG subpopulations presented by the maternal blood but no IgM or IgA.

There are three classes of antibodies in poultry species namely IgG (IgY), IgM and IgA. Poultry IgA and IgM are similar to mammalian IgA and IgM in terms of molecular weight, structure and immunoelectrophoretic mobility **(Lesile & Clem, 1969)**. Although, structural differences exist between IgY and mammalian IgG, IgY is considered the avian equivalent to mammalian IgG **(Hamal et al., 2006).** Additionally, hen's egg yolk contains at least two antigen-binding subclasses of IgG that are derived from the hen's serum and transmitted to the chick, however, IgM and IgA, absent in yolk and in newly hatched chick serum, were detected in the white of infertile eggs as a result of mucosal secretion in the oviduct, in the amniotic fluid of embryonating eggs and in the digestive tract of 19- day embryos, which also contained IgG **(Rose et at., 1974)**.

Avian OrthoAvulaVirus 1 (AOAV-1) (formerly designated as Avian avulavirus 1 (AAvV-1)), commonly known as Avian paramyxoviruses 1 (APMV-1) or Newcastle disease viruses (NDV) cause infections in a wide range of domestic and wild birds worldwide **(ICTV, 2019)**. NDV genotype VII viruses are involved in fatal infections in poultry and other susceptible birds, and they have been responsible for the fourth major panzootic of ND worldwide that continue to the present and is caused by viruses of genotype VII sub genotypes (a, d, b, i). Among genotype VII viruses, sub-genotype VIIi has demonstrated an intercontinental spread and therefore has a global significance in the perspective of potential fifth panzootic **(Miller et al., 2015)**.

In Egypt, from the recent outbreak in poultry flocks, NDV that belongs to class II, genotype VII sub genotype VIIb and subgenotype VIId was isolated **(Moharam et al., 2019)**.

The chicken-origin NDV causes high mortality up to 100% and severe clinical signs, mainly in naive gallinaceous birds **(Miller and Koch, 2013)**. NDV in fully susceptible chickens is generally devastating with mortality up to 100% **(OIE, 2018a)**. Egyptian chicken-origin NDV class II, genotype VIId strain developed severe depression, respiratory and nervous signs post challenge and mortality reached 100% in non-vaccinated infected chicken **(Bastami et al., 2018; Mahmoud et al., 2019)**.

Serological tests like HI allow rapid identification of most of samples and are reliable, sensitive, specific and more accurate methods to detect the viruses for the confirmatory diagnosis of disease **(Rakibul et al., 2010).** HI test, which have already been proven to be the most sensitive ones for monitoring rHVT-F vaccination in chickens **(Gardin et al., 2015; El Khantour et al., 2017)**, were used for the monitoring of humoral immune response. HI test is still the most widely used conventional serological method for measuring anti-NDV antibody levels in poultry sera, and it is considered the standard laboratory test for this disease **(Jestin et al., 1989)**.

The AGID is a simple and economical serological test. AGID does not require specialized laboratory equipment and is commonly used to screen poultry flocks for avian influenza virus infection **(Jenson, 2014)**. The AGID was specific but lacked sensitivity as mentioned by **Robbe-Austerman, et al., (2006)** who discussed why not all PCR positive samples are positive with AGID. The AGID consistently identified two different populations of infected sheep with only moderate overlap between positive test results **(Robbe-Austerman, et al., 2006)**

CONCLUSION

In this study, there are considerable titers of IgY produced from immunized quails against NDV genotype VII. Further studies are under evaluation for the use of such antibodies in therapeutic and/or protective applications.

Conflict of interest

None.

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