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IGY ANTIBODIES FROM EGGS DERIVED FROM DIFFERENT BIRD SPECIES AGAINST H5N1, H9N2 AND NDV

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ABSTRACT

IgY is the major antibody produced by the B cells of domestic birds. It is functionally comparable to mammalian IgG but differs in their structure. Because of its production in large amount and easy way, IgY becomes desirable to be used in both therapeutic and diagnostic approachs in chickens and other bird species. Here, we tested the neutralization ability and efficiency of IgY collected from different bird species, chicken, quail and duck, against avian influenza virus H5N1 and H9N2 as well as Newcastle disease virus. The results showed that IgY collected from different bird species were in high titer and can neutralize the viruses efficiently, exhibiting the ability of using the IgY antibodies as a prophylactic therapy against different viral diseases.

KEYWORDS: H5N1, H9N2, NDV, IgY, Heamaglutination inhibition, ELIZA.

INTRODUCTION

In birds, maternal antibodies are transmitted to their offspring by depositing the antibodies in the egg **(Brambell, 1970)**. In chickens, there are three classes of antibodies namely IgY (IgG), IgA, and IgM. Since the early 1980s, scientists detected IgY produced in birds and some reptiles **(Klemperer, 1893; Warr et al., 1995)**. Chicken IgA and IgM are similar to mammalian IgA and IgM **(Leslie and Clem, 1969)**. IgY is functionally similar to mammalian IgG but differs in their structure **(Michael et al., 2010)**. The egg yolk is rich in IgY **(Leslie and Clem, 1969)**, as the IgY is secreted into chicken's blood and accumulates in the yolk at high concentration **(Narat, 2003; Schade et al., 1996)**, while IgA and IgM are present in the egg white due to mucosal secretion in the oviduct **(Rose et al., 1974)**. The antibodies produced by hens are greater in amount compared to other animals, reducing the number of animals required for the production of antibodies **(Narat, 2003; Schade et al., 1996)**. A single hen lays about 325 eggs per year, and the average egg contains 60 milligrams of IgY protein, meaning that about 20 grams per year of IgY can be recovered from one hen **(Pauly et al., 2011)**. Moreover, the specific IgY produced in chickens are about 1–10% of the total amount of antibodies **(Michael et al., 2010)**. IgY has been broadly used as therapeutic and diagnostic tool. It does not react with the human immune system and shows greater avidity for mammalian conserved proteins **(Pereira et al., 2019)**. Recently, IgY technology has been used for several purposes in human and veterinary health, such as in immunodiagnostics **(Cai et al., 2012)**, immunotherapy **(Rahman et al., 2013)**, neutralization of toxins from venomous animals **(Mendoza et al., 2012)** and bacteria **(LeClaire et al., 2002)**, and as functional food **(Horie et al., 2004)**. The IgY have been used for treatment and prevention of different bacterial and viral infections. Oral administration of egg yolk powder rich in IgY against bovine group A Rotavirus disease lead to attenuation of the infection in treated animals **(Vega et al., 2015)**. IgY against the S1 protein of porcine epidemical diarrhea virus orally administered in piglets reduced the severity of diarrhea and intestinal lesions and abolished the mortality of piglets due to the disease **(Lee et al., 2015)**. The IgY has been also used against dengue fever and neutralized the virus *in-vitro* and *in-vivo* **(Ashley L Fink et al., 2017)**. Studies performed on IgY against influenza virus H1N1, H3N2 and H5N1 indicated that IgY could be used in nasal, oral or spray applications to protect individuals and environments **(Michael G Wallach et al., 2011)**. Recently, IgY targeting S1 subunit of SARS-CoV-2 blocked the entry of the virus making it a promising candidate for pre- and post-exposure prophylaxis or treatment of COVID-19 **(Bao et al., 2022)**. In a previous study, significant titers of IgY against NDV were detected and could efficiently produce protective IgY against NDV field isolates **(Hezema et al., 2020)**. On the other hand, intranasal administration of IgY against H5N1 one hour prior to infection protected 100% of mice against H5N1 in an in vivo study and protected against H1N1 both in vitro and in vivo **(M. G. Wallach et al., 2011)**. Here, we aimed to show the neutralization efficiency of IgY collected from different bird species as chicken, quail and duck against the avian influenza viruses H5N1 and H9N2 as well the

Newcastle disease virus. The results emphasize the ability of IgYs to neutralize different viruses efficiently.

MATERIAL AND METHODS

1. Samples

Eggs from duck, quail and chicken were collected from 19, 19 and 63 eggs, respectively. Duck eggs were collected from house bred ducks, chicken and quail eggs were purchased from commercially sold eggs. Egg yolk was collected for further purification.

2. Viruses

Inactivated reassortant H5N1 and inactivated H9N2 vaccines obtained from MEVAC (Middle East for Veterinary Vaccines, Egypt) and NDV live attenuated virus obtained from lasota vaccine was used as the antigen in the HI test. The standard amount of viruses used in the haemagglutination inhibition (HI) test was 4HA units **(Kaufmann et al., 2017)**.

3. Extraction of IgY

Two mL of yolk and 2 mL of Phosphate-Buffered Saline (PBS) were mixed well. Two volumes of chloroform were added to one volume of the mixture. The mixture was incubated for 1 hr at room temperature. Following centrifugation at 383×g for 20 min, the supernatant was collected and stored at -20°C **(Abolfazl et al., 2017).**

4. Haemagglutination- inhibition (HI) test

HI test was done using U-shape microtiter plate and 1% chicken RBCs against 4HA units of virus antigens according to **OIE, (2015).**

5. Enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assay (ELISA) **(BioCheck UK Ltd.)** was carried out on standard 96-well flat bottom microtiter plates for testing H5N1, H9N2 and NDV according to manufacturer's instructions and according to **Molesti et al., (2012)**.

RESULTS

Titers of IgY derived from duck eggs against H5N1, H9N2 and NDV:

From 19 egg yolk containing IgY from eggs derived from ducks, the HI titer was detected in 15 (78.9%), 19 (100%) and 14 (73.7%) samples against H5N1, H9N2 and NDV, respectively **(Table 1)**. Four samples had titers against AIV with no titers against NDV, three samples had no titers against H5N1 and had titers against H9N2 and NDV, and one sample had titer only for H9N2 and had no titers against H5N1 and NDV, while other samples gave titers against the three viruses **(Figure 1)**. The HI titers against H5N1 and H9N2 viruses ranged from 2^4 to 2^{10} while, the HI titers against the NDV ranged from 2^4 to 2^9 **(Figure 1)**.

The antibody titer was also measured by ELIZA against the common antigen for avian influenza and Newcastle disease virus. The results showed that all 19 samples (100%) had titers against influenza virus and 14 out of 19 samples (73.7%) had titers against NDV **(Table 1 and Figure 2)**. The antibody titer for AIV ranged from 1473 to 10279, while for NDV ranged from 2000 to 7420 **(Table 1).**

These results showed that all HI negative NDV samples were ELIZA negative, while in case of AIV all samples were positive by ELIZA even with the HI negative for H5N1.

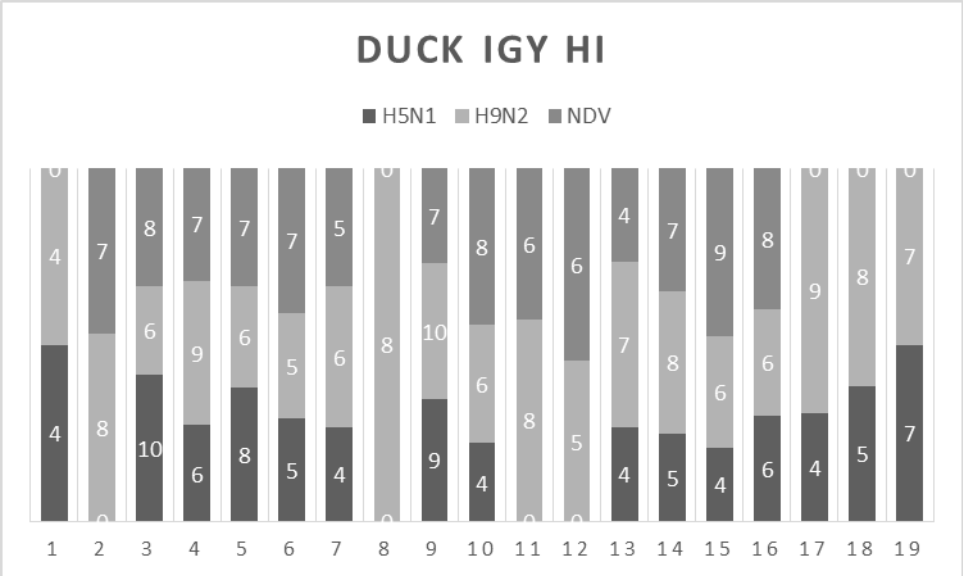

Figure (1): HI titers against H5N1, H9N2 and NDV in duck eggs.

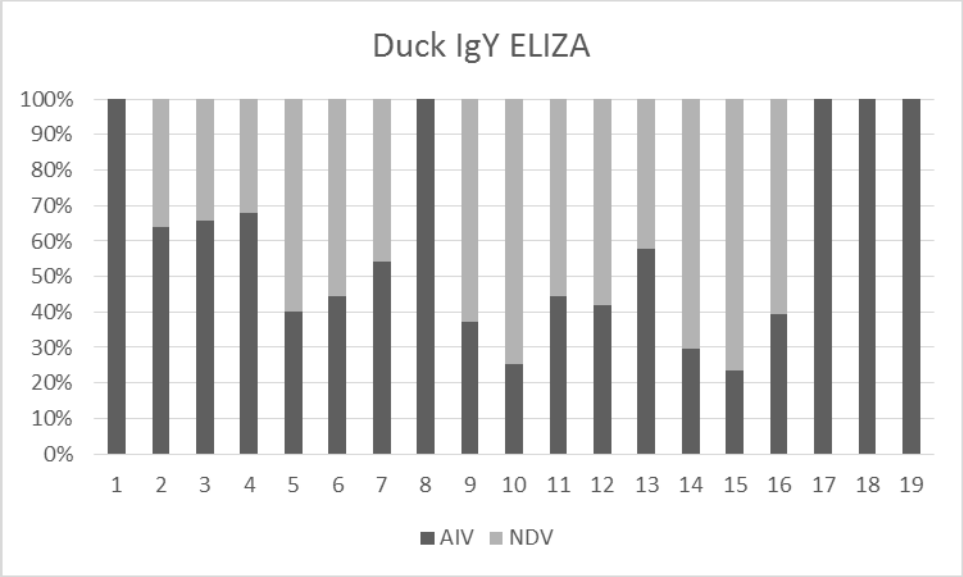

Figure (2): ELIZA titers against AIV and NDV in duck eggs.

Table 1: HI and ELIZA titers against H5N1, H9N2 and NDV in duck eggs.

Sample No.	Avian influenza (AIV)			New castle disease virus (NDV)	
	HI test		ELISA test	HI test	ELISA test
	H5N1	H9N2			
1	2^4	2^4	2101	0	0
2	0	2^8	3651	2^7	2051
3	2^{10}	2^6	10279	2^8	5384
4	2^6	2^9	6374	2^7	3013
5	2^8	2^6	2046	2^7	3062
6	2^5	2^5	2402	2^7	3010
7	2^4	2^6	2460	2^5	2070
8	0	2^8	2260	0	0
9	2^9	2^{10}	1819	2^7	3070
10	2^4	2^6	1473	2^8	4384
11	0	2^8	2402	2^6	3013
12	0	2^5	2173	2^6	3005
13	2^4	2^7	2734	2^4	2000
14	2^5	2^8	1834	2^7	4325
15	2^4	2^6	2259	2^9	7420
16	2^6	2^6	3409	2^8	5240
17	2^4	2^9	5324	0	0
18	2^5	2^8	5032	0	0
19	2^7	2^7	4320	0	0
Total	15/19 (78.9%)	19/19 (100%)	19/19 (100%)	14/19 (73.7%)	14/19 (73.7%)

Titers of IgY derived from quail eggs against H5N1, H9N2 and NDV

From 19 egg yolk containing IgY from eggs of quails, the HI titer was detected in 17 samples (89.5%) against H5N1, H9N2 and NDV **(Table 2)**. One sample had no titer against NDV but had titers against H5N1 and H9N2, one sample had no titer against H5N1 and H9N2 viruses but had titer against NDV, and one sample had no titer against the three viruses, while other samples had HI titers for the three viruses **(Figure 3)**. The HI titers against the H5N1 ranged from 2^1 to 2^{11}, while the titers against H9N2 ranged from 2^1 to 2^{10}, and the HI titers against NDV ranged from 2^4 to 2^{10} **(Figure 3)**.

The antibody titer was measured by ELIZA against the common antigen for avian influenza and Newcastle disease virus. The results showed that 17 out of 19 samples (89.5%) had titers against influenza virus and NDV **(Table 2 and Figure 4)**. The antibody titer for AIV ranged from 5040 to 20075 and antibody titer for NDV ranged from 3240 to 15249 **(Table 2).**

The results of AIV showed that one sample was positive HI and negative ELIZA and another sample was positive ELIZA and negative HI. In contrast, the results of NDV showed that two samples were negative HI and ELIZA. One sample showed no titer against AIV and NDV by both HI and ELIZA test.

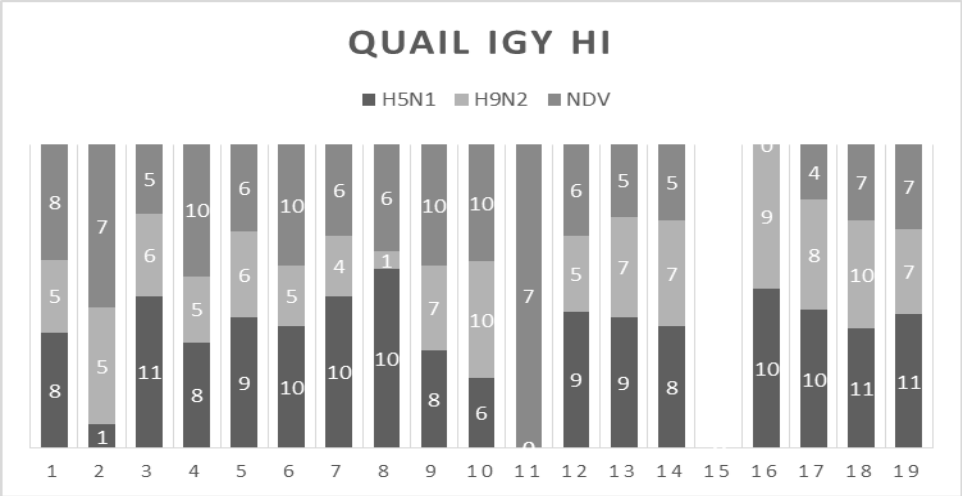

Figure (3): HI titers against H5N1, H9N2 and NDV in quail eggs.

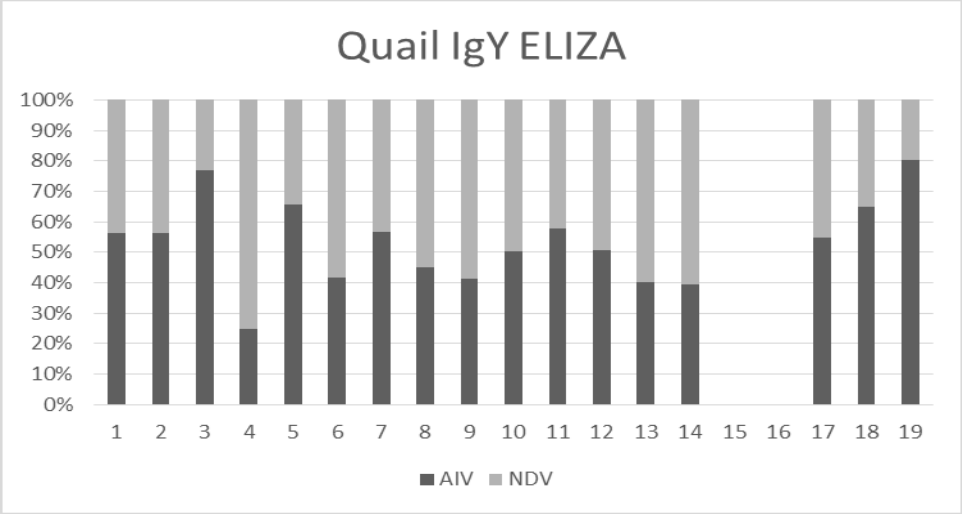

Figure (4): ELIZA titers against AIV and NDV in quail eggs.

Table (2): HI and ELIZA titers against H5N1, H9N2 and NDV in quail eggs.

Sample No.	Avian influenza (AIV)			New castle disease virus (NDV)	
	HI test		ELISA test	HI test	ELISA test
	H5N1	H9N2			
1	2^8	2^5	14562	2^8	11245
2	2^1	2^5	16982	2^7	13201
3	2^{11}	2^6	20075	2^5	6014
4	2^8	2^5	5040	2^{10}	15249
5	2^9	2^6	13618	2^6	7159
6	2^{10}	2^5	6094	2^{10}	8538
7	2^{10}	2^4	8637	2^6	6609
8	2^{10}	2^1	7339	2^6	8976
9	2^8	2^7	7261	2^{10}	10289
10	2^6	2^{10}	9745	2^{10}	9687
11	0	0	10582	2^7	7687
12	2^9	2^5	8460	2^6	8241
13	2^9	2^7	6852	2^5	10241

14	2^8	2^7	7339	2^5	11240
15	0	0	0	0	0
16	2^{10}	2^9	0	0	0
17	2^{10}	2^8	11214	2^4	9245
18	2^{11}	2^{10}	9570	2^7	5210
19	2^{11}	2^7	13262	2^7	3240
Total	17/19 (89.5%)	17/19 (89.5%)	17/19 (89.5%)	17/19 (89.5%)	17/19 (89.5%)

Titers of IgY derived from chicken eggs against H5N1, H9N2 and NDV

From 63 egg yolk containing IgY from eggs of chickens, the HI titer was detected in 61 (96.8%), 59 (93.7%) and 52 (82.5%) samples against H5N1, H9N2 and NDV, respectively **(Table 3)**. Nine samples had no titers against NDV, one sample had no titer against H5N1, two samples had no titer to H9N2, one sample had titer only for H5N1, while other samples gave titers to the three viruses **(Figure 5)**. The HI titers against H5N1 virus ranged from 2^1 to 2^{11}, while, the HI titers against NDV and H9N2 ranged from 2^1 to 2^{10} **(Figure 5).**

The antibody titer was confirmed by ELIZA against the common antigen for avian influenza and Newcastle disease virus. The results showed that 59 out of 63 (93.6%) samples had titers against influenza viruses and 56 out of 63 (88.9%) had titers against NDV **(Table 3 and Figure 6)**. The antibody titer for AIV ranged from 1570 to 29495 while the titer for NDV ranged from 581 to 17259 **(Table 3)**.

The results of AIV showed that three samples were positive HI and negative ELIZA. In contrast, the results of NDV showed that four samples were negative HI and positive ELIZA and six samples were negative both HI and ELIZA. One sample showed no titer against AIV and NDV by both HI and ELIZA test.

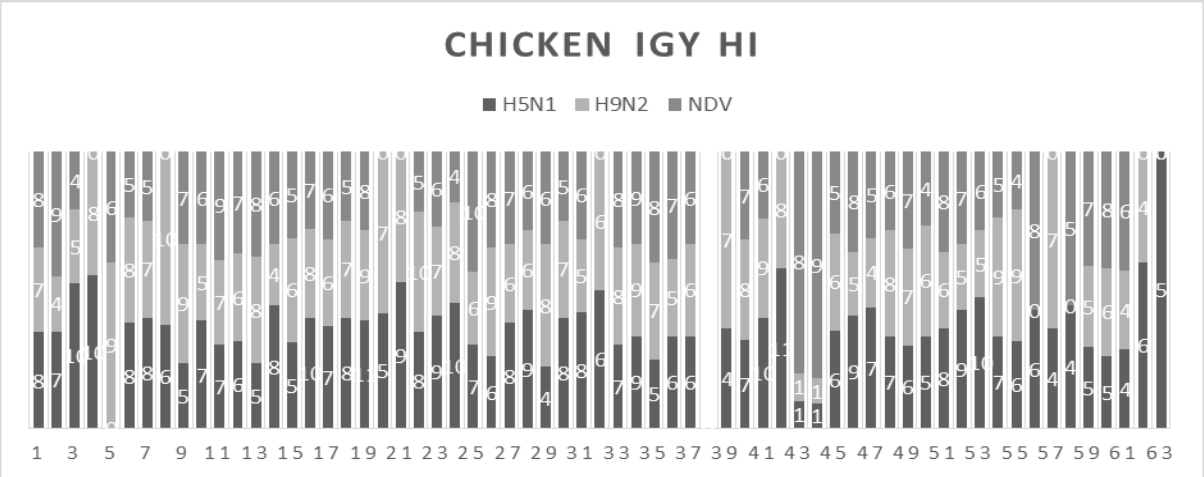

Figure (5): HI titers against H5N1, H9N2 and NDV in chicken eggs

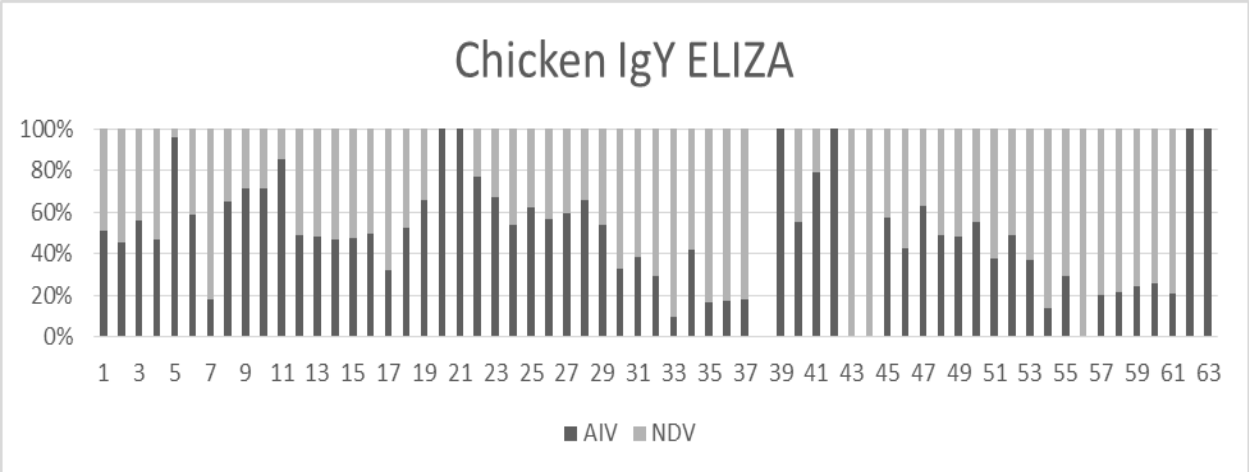

Figure (6): ELIZA titers against AIV and NDV in chicken eggs.

Table (3): HI and ELIZA titers against H5N1, H9N2 and NDV in chicken eggs.

Sample No.	Avian influenza (AIV)			New castle disease virus (NDV)	
	HI test		ELIZA test	HI test	ELISA test
	H5N1	H9N2			
1	2^8	2^7	5432	2^8	5252
2	2^7	2^4	3214	2^9	3878
3	2^{10}	2^5	6991	2^4	5460
4	2^{10}	2^8	4789	0	5460
5	0	2^9	13204	2^6	581
6	2^8	2^8	6820	2^5	4831
7	2^8	2^7	1570	2^5	7189
8	2^6	2^{10}	17600	0	9278
9	2^5	2^9	14376	2^7	5699
10	2^7	2^5	14917	2^6	5907
11	2^7	2^7	29495	2^9	4891
12	2^6	2^6	6189	2^7	6503
13	2^5	2^8	4933	2^8	5222
14	2^8	2^4	5395	2^6	6146
15	2^5	2^6	2647	2^5	2894
16	2^{10}	2^8	9801	2^7	9804
17	2^7	2^6	4876	2^6	10256
18	2^8	2^7	6587	2^5	5907
19	2^{11}	2^9	13246	2^8	6861
20	2^5	2^7	3618	0	0
21	2^9	2^8	9886	0	0
22	2^8	2^{10}	8625	2^5	2507
23	2^9	2^7	4523	2^6	2209
24	2^{10}	2^8	5214	2^4	4503
25	2^7	2^6	8435	2^{10}	5103
26	2^6	2^9	6872	2^8	5162
27	2^8	2^6	7459	2^7	5103
28	2^9	2^6	9524	2^6	4921
29	2^4	2^8	7304	2^6	6265
30	2^8	2^7	2331	2^5	4809
31	2^8	2^5	2088	2^6	3287
32	2^6	2^6	2473	0	5893
33	2^7	2^8	1819	2^8	17259
34	2^9	2^9	1792	2^9	2453
35	2^5	2^7	1890	2^8	9583
36	2^6	2^5	1626	2^7	7509
37	2^6	2^6	2509	2^6	11201
38	0	0	0	0	0
39	2^4	2^7	4692	0	0
40	2^7	2^8	7206	2^7	5809
41	2^{10}	2^9	9684	2^6	2540
42	2^{11}	2^8	8410	0	0
43	2	2	0	2^8	8083
44	2	2	0	2^9	3460
45	2^6	2^6	5318	2^5	3937
46	2^9	2^5	6301	2^8	8563
47	2^7	2^4	8142	2^5	4801
48	2^7	2^8	3982	2^6	4176
49	2^6	2^7	5907	2^7	6295
50	2^5	2^6	7268	2^4	5907
51	2^8	2^6	4158	2^8	6921
52	2^9	2^5	3782	2^7	3967
53	2^{10}	2^5	5217	2^6	8861

54	2^7	2^9	2395	2^5	14765
55	2^6	2^9	1890	2^4	4474
56	2^6	0	0	2^8	6682
57	2^4	2^7	1736	0	6712
58	2^4	0	1863	2^5	6682
59	2^5	2^5	2144	2^7	6712
60	2^5	2^6	2402	2^8	6861
61	2^4	2^4	2144	2^6	8053
62	2^6	2^4	2301	0	0
63	2^5	0	2159	0	0
Total	61/63 (96.8%)	59/63 (93.7%)	59/63 (93.7%)	52/63 (82.5%)	56/63 (88.9%)

DISCUSSION

Recently, many technologies associated with using IgY have emerged. IgY technology has been introduced in a large number of valuable immunochemical tools for biotechnology and medicine since the 1990s. Laying hens are used for producing the IgY antibodies. Other types of poultry such as duck, goose, ostrich, and quail have been used to a lesser extent **(Adachi et al., 2008; Brocato et al., 2012; Esmailnejad et al., 2019; Haese et al., 2015)**. Quail, ostrich and other avian species may provide auxiliary advantages in the field of IgY technology, such as convenient housing and breeding conditions for quails or exceptionally high amounts of IgYs obtained for ostrich. These specific IgYs have been developed and used for treatment of the recent pandemic COVID-19 **(Constantin et al., 2020; Lu and Wang, 2020; Pérez de la Lastra and Baca-González, 2020)** and other diseases. In this study, the IgY antibody was extracted and purified from egg yolks of different avian species as chicken, quail and duck. These birds were commercially used for meat production, we aimed to study the efficiency of IgY extracted from the egg of that birds to detect the common viral infections as avian influenza virus; H5N1 and H9N2 as well as NDV. The IgY titers were detected by different methods like HI and ELIZA to confirm the results. The IgY collected from different egg species showed comparable detection efficiency against avian influenza virus H5N1, H9N2 and NDV. While the results of ELIZA showed higher IgY titers than HI in different eggs obtained from chicken, quail and duck. These results agreed with the results showing that ELISA is more sensitive than HI for diagnosis of rubella virus **(Vejtorp and Leerhoy, 1980)**. The ELISA was able to detect antibody levels at least ten times lower than the HI and was less cross-reactive than HI **(Williams et al., 1997)**. In a previous study that compared between ELIZA and HI tests for the detection of NDV antibodies in human sera, the results revealed that 22 % more sera were positive by ELISA as compared to HI. No samples were negative by ELISA that was more sensitive and the titers obtained by ELISA were nearly six times higher than those by HI **(Charan et al., 1981)**. Moreover, ELIZA was 93.6% sensitive and 98.7% specific relative to HI test in the detection of egg drop syndrome virus. ELISA was efficacious in quantification of both vaccinal and infection antibodies and could routinely be used for screening large numbers of field sera **(Raj et al., 2004)**. The IgY produced in the egg yolk of different bird species have high efficiency to detect different viruses as H5N1 and H9N2 avian influenza and NDV as shown by the results here. These findings comes in agreement with the results obtained from different studies that IgY had been used for the diagnosis and detection of some viral and bacterial infections. The IgYs were used in ELISA and immunochromatography in the detection of canine parvovirus in dog fecal samples showing sensitivity and specificity **(He et al., 2015)**. Moreover, IgY Specific antibodies were used in ELISA to detect bovine viral diarrhea virus that cause diarrhea in cattle in serum samples of cows with diarrhea, showing a concordance of 95, 45% and 90% with RT-PCR, respectively **(Zhang et al., 2016)**. IgY also were used to detect hepatitis A virus showing high sensitivity and specificity **(Silva et al., 2012)**. In addition, Anti-NP IgY was used in ELISA for the diagnosis of acute respiratory syndrome associated to coronavirus (SARS-CoV) and it shows high sensitivity enough to detect small amounts of NP protein **(Palaniyappan et al., 2012)**. IgY was also able to be used in diagnosis of the dengue fever virus as described previously **(Figueiredo et al., 2015)**. On the other hand, IgYs had been used for diagnosis of bacterial infections as *Staphylococcus aureus* as described by different studies **(Mudili et al., 2015; Reddy et al., 2013; Richman et al., 1982; Walczak et al., 2015; Yamada et al., 2013)**. The IgY antibodies have the ability to neutralize the MERS-CoV and SARS-CoV in Vero cells and blocked the SARS-CoV-2 pseudovirus **(Abbas et al., 2020; Fu et al., 2006; Wei et al., 2021)**. The IgY protected challenged mice with lethal Zika virus **(O'Donnell et al., 2019)** and lethal dengue virus (DENV2) **(A. L. Fink et al., 2017; O'Donnell et al., 2020)**. It also reduced the incidence and duration of human rota virus diarrhea in suckling mice and neutralized the bovine rota virus in Vero cells **(Hatta et al., 1993; Kim et al., 2017; Odagiri et al., 2020; Rahman et al., 2012)**. Besides, the IgY neutralized the effect of enteroviruses in mice **(Liou et al., 2010)** and showed protective efficacy of Ebola virus in mice **(Zhang et al., 2021)** as well as its neutralization efficiency against the bovine respiratory syncytial virus in MDBK cells **(Ferella et al., 2012)**. Moreover, it exhibited ability to reduce the rabbit infection against

rabbit hemorrhagic disease virus challenge **(Li et al., 2014)**. The previous studies showed that IgY antibodies can reduce the infectious titer of H1N1 virus in the mice lung and neutralize the viral infectivity in MDCK cells as well as reducing the mice infection against lethal challenge with H5N1 and neutralizing of viral infectivity in MDCK cells **(Adachi et al., 2008; Tsukamoto et al., 2011; M. G. Wallach et al., 2011; Yang et al., 2014)**.

This study revealed the ability and efficiency of IgY obtained from chicken, quail and duck in detection of avian influenza virus H5N1 and H9N2 and NDV. Further studies to show the ability of these antibodies in protection against the viruses in vivo and as a prophylactic therapy are needed to explore the importance of IgYs in the modern therapy against different infections either bacterial or viral.

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