The Relation between some Immunosuppressive Agents and Widespread Nature of Highly Pathogenic Avian Influenza (HPAI) Post Vaccination

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Abstract: The effect of chicken infectious anemia virus (CIAV) and mycotoxins on immune response of chicken after vaccination against highly pathogenic avian influenza was evaluated. Sixteen chicken flocks (4 broiler flocks ranged between 4 - 6w old and 12 layer flocks ranged between 12 - 57w old) vaccinated against HPAI once in case of broiler flocks and three times in case of layer flocks showed non protective titer by HI and ELISA tests, were tested for the presence of Anti-CIAV antibody using commercially available ELISA kit and the flock's rations were examined for the presence of aflatoxin and ochratoxin using HPLC. All tested flocks were seropositive against CIAV in both broiler flocks (with percentage of 70% & ELISA titers ranging from 2105 to 3728) and layer flocks (with percentage of 71.67% & ELISA titers ranging from 2007 to 3194) of different ages, breeds, and localities in Sharkia province, Egypt. HPLC analysis revealed the presence of aflatoxin & ochratoxin residues in rations despite using antimycotoxin feed additives. The study revealed that CIAV infection and mycotoxicosis might be the cause of vaccination failure against AIV and so the repeated occurrence of AIV infection even in the vaccinated flocks in Sharkia province, Egypt.
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1. Introduction:

Highly pathogenic avian influenza (HPAI) was recognized as a global problem, especially in certain Africa countries (*Cattoli et al., 2009*). World authorities concern that an HPAI H5N1 strain would mutate causing pandemic outbreak in humans. To decrease and prevent that possibility, a vaccination program was enforced nationwide as well as depopulation of affected poultry farms had been conducted in infected areas at the beginning of HPAI outbreaks in Africa in early 2006 as recommended by the Office International des Epizooties. However, such program could not successfully eradicate HPAI from the African countries and so HPAI cases occurred repeatedly in some vaccinated flocks, perhaps because of cultural and economic reasons in the implementation of such program. Although vaccination induced satisfactory antibody reaction to H5N1 experimentally, some vaccinated flocks demonstrated poor, or no, antibody response, especially after one vaccination in young flocks (*Jiang et al., 2005*).

Chicken infectious anemia virus (CIAV) was first isolated in 1979 in Japan *(Yuasa et al., 1979).* Since then, an increasing interest was paid to that virus, as it was found to have a great economic impact on poultry industry. The virus was incriminated in a disease of young chickens, characterized by a transient severe destruction of erythrocytic and granulocytic series of the bone marrow cells, resulting in aplastic anemia. It also caused severe depletion of lymphocytes from primary and secondary lymphoid organs, resulting in immunosuppression (*Yuasa et al., 1979; Taniguchi et al., 1982 & 1983)*, giving reason for more severe secondary infections and inadequate response to vaccines *(Novak et al., 2001; Shuhong et al., 2009).* CIAV was proved to participate other immunosuppressive viruses such as infectious bursal disease virus (IBDV) *(Yuasa et al., 1980)*, Marek's disease virus (MDV) and reticuloendotheliosis virus (REV) *(Bülow et al., 1986).* It also enhanced the pathogenicity of a wide range of co-infecting pathogens such as Newcastle disease virus *(De Boer et al., 1994)*, Marek's disease virus *(Miles et al., 2001)*, Adenovirus *(Toro et al., 2000)*, Reovirus *(McNeilly et al., 1995).*

Immunity acquired through vaccination is also impaired by mycotoxins ingestion. The important mycotoxins in poultry rations were aflatoxin and ochratoxin *(Girish and Smith, 2008).* Mycotoxin-induced immunosuppression may be manifested as depressed T- or B-lymphocyte activity, suppressed antibody production and impaired macrophage/neutrophil-effector functions *(Hatori et al., 1991; Mohiuddin, 1992).* Suppressed immune function by mycotoxins might eventually decrease resistance to infectious diseases, reactivate chronic infections and/or decrease vaccines efficacy *(Oswald et al., 2005).* Therefore, the presence of mycotoxins in poultry rations might lead to a breakdown in vaccinal immunity and to the occurrence of diseases

even in properly vaccinated flocks *(Hashad, 1991; Pier, 1992)* such as infectious bursal disease virus (IBDV) *(Somvanshi and Mohanty, 1991)* and Adenovirus *(Shivachandra et al., 2003)*. Low levels of toxins -below observable overt toxicity- in rations were also likely to alter normal immune functions *(Smith and Ross, 1991; Verma et al., 2004)*.

Our study aimed at evaluation of the role of chicken infectious anemia virus (CIAV) infection and mycotoxicosis in reduction of vaccinal immunity against highly pathogenic avian influenza virus (HPAI) infection and so the widespread of AIV infection even in vaccinated flocks in Sharkia province, Egypt.

2. Material and Methods

Samples collection & storage

A total of 240 serum samples from 16 poultry flocks (12 from commercial layer flocks ranged in age from 12 to 57w and 4 from commercial broiler flocks ranged in age from 4 to 6w) representing different localities in Sharkia province, Egypt were collected and stored at (- 70°C) until used. All flocks were vaccinated against AIV (inactivated H5N1 oil emulsion vaccines) once or more. None of the flocks were vaccinated against CIAV.

Reference Virus

Local virus isolate chicken/Egypt/2008 (H5N1) with EID 50 of $10^{7.56}$/0.1ml was obtained from Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University.

Reference AIV Antiserum

Anti-avian influenza hyperimmune serum (H5N1 antiserum chicken/Egypt/2008), was obtained from Department of Avian and Rabbit Medicine, Faculty of Veterinary Medicine, Zagazig University.

Washed chicken RBCs suspension

Blood was collected after slaughtering chickens. 5ml chicken blood was received in sterile tubes containing anticoagulant sodium (citrate solution 3.8%). The blood was centrifuged at 1500 rpm for 15 minutes, RBCs were washed three times in PBS, stored at 4°C as 10% stock solution. For haemagglutination inhibition test (HI) test, RBCs were used as 1 % suspension in saline. (*OIE, 2005*)

Phosphate buffer saline (PBS)

The solution was prepared according to *(Voller et al., 1976)* .The pH was adjusted to 7.4 and kept at 4°C till used in HA and HI testes.

Hemagglutination and Hemagglutination Inhibition (HI) tests of AIV

Using of U-bottomed microtiter plastic plates and 1% washed chicken RBCs suspension is the recommended method by OIE, (2005). The mean HI titer (Log2) was calculated and expressed in geometric mean titer (GMT). *(Brugh, 1978).*

Enzyme-Linked Immunosorbent Assay (ELISA)

A- For AIV:

Commercially available H5 avian influenza (AIV) antibody ELISA test kit (ProFLOK® PLUS, Synnbiotics Corporation, San Diego, CA, USA) was used under the manufacturer instructions.

B. For CIAV:

Commercially available chicken anemia virus (CIAV) antibody ELISA test kit **(ProFLOK® PLUS, Synnbiotics Corporation, San Diego, CA, USA)** was used under the manufacturer instructions. Optical density values were read at 450 nm using using ELISA reader **(Behring EL311)**.

Estimation of mycotoxins in rations

Aflatoxin and Ochratoxin residues estimated by using HPLC (water model 501 solvent delivery system) as described by *(Saqer, 2009).*

Statistical analysis of the data

Sensitivity, specificity and agreement between HI and ELISA tests used to estimate the humoral immunity against AIV vaccine were performed using Microsoft office Excel 2007 program **(Microsoft, USA).**

3. Results

Hemagglutination inhibition (HI) result of AIV

Geometric mean titers (log 2) of haemagglutination inhibition (HAI) test for vaccinated chicken with H5N1 oil emulsion vaccine was estimated as shown in **(Table 1).**

ELISA test for antibody detection against avian influenza virus

Evaluation of immune response of vaccinated commercial layer and broiler chicken flocks vaccinated by inactivated oil emulsion vaccine H5N1 using ELISA was shown in **(Table 2).** The positive antisera showed 0.4 OD which is considered +ve for ELISA.

Sensitivity, specificity and agreement between HI and ELISA tests

An assay of 240 serum samples for AIV antibodies showed that a commercial indirect AIV ELISA had 94.2 % sensitivity and 88.9 % specificity relative to AIV HI test. The ELISA values regressed significantly on the HI titers. The correlational coefficient was 0.85. The agreement between the ELISA and the HI test was calculated to be 0.84, which indicates a highly significant agreement between the two tests.

Seroprevalence of CIAV infection in commercial broiler and layer chicken using indirect ELISA

The results of ELISA showed that all flocks of different ages were positive for anti-CAV antibodies. Rates of seropositive chicken among flocks ranged from 40% to 100% as shown in **(Table 3).**

Quantitative estimation of aflatoxin and ochratoxin residues using HPLC

The present levels of aflatoxin and ochratoxin in examined flocks was shown in **(Table 4).**

Table (1): Distribution of vaccinated layer and broiler chicken flocks on the basis of log2 HI titers obtained against AIV- subtype H5

Flock No.	Flock breed*	Age (W)	Sera No.	Positive sample No.	Antibody titers using HI test								
					1:2	1:4	1:8	1:16	1:32	1:64	1:12	1:25	GMT
1	Cobb (B)	4	15	14	-	1	3	6	2	-	1	1	16
2	Cobb (B)	6	15	14	6	3	5	-	-	-	-	-	3.8
3	Cobb (B)	6	15	12	3	2	2	1	3	1	-	-	6.56
4	Cobb (B)	4	15	15	2	5	3	5	-	-	-	-	6.64
5	Lohmann (L)	40	15	0	-	-	-	-	-	-	-	-	0
6	Lohmann (L)	48	15	0	-	-	-	-	-	-	-	-	0
7	Saso (L)	12	15	15	5	3	5	1	1	-	-	-	5.03
8	Balady (L)	44	15	15	1	3	2	2	6	1	-	-	13.9
9	Lohmann (L)	40	15	14	1	4	3	2	3	1	-	-	10.2
10	Balady (L)	12	15	0	-	-	-	-	-	-	-	-	0
11	Hisex (L)	40	15	14	-	3	4	2	6	-	-	-	13.2
12	Balady (L)	50	15	0	-	-	-	-	-	-	-	-	0
13	Balady (L)	57	15	15	-	1	2	5	1	5	-	-	22.6
14	Saso (L)	40	15	15	2	5	4	1	3	-	-	-	7.2
15	Balady (L)	54	15	15	1	-	2	3	6	3	-	-	22.1
16	Lohmann (L)	50	15	15	-	-	5	5	5	-	-	-	16

*B: broiler, L: layer

Table (2): Immune response of layer and broiler chicken flocks post vaccination with AIV inactivated oil-emulsion vaccines H5N1 using indirect ELISA

Flock No.	Flock breed	Age (weeks)	Sera No.	AIV-ELISA		
				Mean Sp ratio	Mean Titers	Condition
1	Cobb (B)	4	15	0.057	26	negative
2	Cobb (B)	6	15	0.163	142	negative
3	Cobb (B)	6	15	0.055	27	negative
4	Cobb (B)	4	15	0.049	22	negative
5	Lohmann(L)	40	15	0.027	9	negative
6	Lohmann(L)	48	15	0.094	61	negative
7	Saso(L)	12	15	0.152	118	negative
8	Balady(L)	44	15	0.247	222	negative
9	Lohmann(L)	40	15	0.214	169	negative
10	Balady(L)	12	15	0.078	48	negative
11	Hisex(L)	40	15	0.053	30	negative
12	Balady(L)	50	15	0.158	120	negative
13	Balady(L)	57	15	0.208	174	negative
14	Saso(L)	40	15	0.414	439	negative
15	Balady(L)	54	15	0.142	109	negative
16	Lohmann(L)	50	15	0.048	19	negative

*B: broiler, L: layer

Table (3): Seroprevalence of CIAV infection in commercial broiler and layer chicken using indirect ELISA

Flock No.	Flock breed*	Age (weeks)	Sera No.	Positive sample No.	positive sample %	CIA-ELISA		
						Mean Sp ratio	Mean Titers	Condition
1	Cobb (B)	4	15	15	100	0.878	3728	positive
2	Cobb (B)	6	15	12	80	0.779	3299	positive
3	Cobb (B)	6	15	6	40	0.498	2105	positive
4	Cobb (B)	4	15	9	60	0.569	2406	positive
5	Lohmann	40	15	12	80	0.446	2533	Positive
6	Lohmann	48	15	10	67	0.523	2212	positive
7	Saso (L)	12	15	8	53	0.690	2920	positive
8	Balady (L)	44	15	12	80	0.475	2007	positive
9	Lohmann	40	15	9	60	0.731	3094	positive
10	Balady (L)	12	15	13	87	0.594	2513	positive
11	Layers/Hise	40	15	10	67	0.743	3149	positive
12	Balady (L)	50	15	13	87	0.548	2315	positive
13	Balady (L)	57	15	10	67	0.532	2319	positive
14	Saso (L)	40	15	10	67	0.529	2236	positive
15	Balady (L)	54	15	12	80	0.591	2498	positive
16	Lohmann	50	15	10	67	0.685	2901	positive

*B: broiler, L: layer

Table (4): Aflatoxin and Ochratoxin levels in examined rations using HPLC

Flock No.	Flock breed*	Age (weeks)	Aflatoxin (ppb)	Ochratoxin (ppb)
1	Cobb (B)	4	0.97	17
2	Cobb (B)	6	0.47	22
3	Cobb (B)	6	5.3	21
4	Cobb (B)	4	5.9	23
5	Lohmann (L)	40	3	20
6	Lohmann (L)	48	0	6
7	Saso (L)	12	1	16
8	Balady (L)	44	1	16
9	Lohmann (L)	40	1	6
10	Balady (L)	12	1	11
11	Layers/Hisex	40	0	6
12	Balady (L)	50	0	18
13	Balady (L)	57	1	18
14	Saso (L)	40	1	6
15	Balady (L)	54	1	9
16	Lohmann (L)	50	0	6

*B: broiler, L: layer

4. Discussion

Avian influenza virus (AIV) remains one of the greatest health concerns for both human and poultry around the world. The greatest concern typically has been for highly pathogenic avian influenza (HPAI) because of its severe clinical disease and its effects on trade. However, low pathogenic avian influenza (LPAI) also remains a concern because of its ability to cause disease and production losses, it is found more widely than HPAI, and for its potential to mutate to HPAI remains ever present *(Swayne and Jackwood, 2006).* Vaccination has become a recommended tool to support the eradication efforts and to limit the economic losses due to AI. However, the virus is still able to replicate in clinically healthy vaccinated birds *(Beato et al., 2007).* The role of some immunosuppressive agents as chicken infectious anemia virus (CIAV) and mycotoxins in vaccination failure and the repeated occurrence of avian influenza virus infection were studied.

Protection level of the tested vaccinated flocks against AIV and the efficacy of vaccination were measured by Hemagglutination Inhibition test (HI) and Enzyme-Linked Immunosorbent Assay (ELISA). Titration of antibody level by HI is the convenient and best technique to measure the level of protection against AIV as well as the efficacy of vaccine *(Ewing et al., 1994).* Antibody levels with GMT value of 67.29 and higher were considered as protective against avian influenza *(Trani et al., 2002).* ELISA is another option for AIV

surveillance and/or evaluation of vaccine efficacy as it is shown to be specific for AIV and does not cross-react with chicken sera that has antibodies to other avian viruses *(Jindal et al., 1994)*. According to the manufacturer of ELISA kit, samples with Sp value of less than 0.35 is considered negative and so the antibody level is not protective against avian influenza.

In the present study, All tested flocks, that were vaccinated once against AIV in case of broiler flocks (Flock 1 to 4) and three times in case of layer flocks (Flock 5 to 16), had GMT values lower than 67.29 **(Table 1)** and Sp value less than 0.35 **(Table 2)** suggesting that they fall in the non protective antibody titer range against AIV-subtype H5N1. The sensitivity and specificity of ELISA and HI test were 94.2% and 88.9%, respectively showing significant correlation (R- value >0.85). The agreement between the ELISA and the HI test was calculated to be 0.84, indicating highly significant agreement between the two tests. Our findings go with the statements of previous researches that indicated that HI and ELISA tests had high agreement ratio and statistically no significant difference *(Meilinjin et al., 2004)* also proved that the tests can be used efficiently for the detection of Abs in the serum samples

CIAV infection causes immunosuppression *(Yuasa et al., 1979; Taniguchi et al., 1982 & 1983)*, giving reason for more severe secondary infections and inadequate response to vaccines *(Novak et al., 2001; Shuhong et al., 2009)*. To explore the role of CIAV in failure of vaccination programs against AI, the serum samples collected from the tested flocks were tested using ELISA kit. The overall serological findings revealed that all tested flocks were seropositive against CIAV in both broiler flocks (with percentage of 70% & ELISA titers ranging from 2105 to 3728) and layer flocks (with percentage of 71.67% & ELISA titers ranging from 2007 to 3194) of different ages, breeds, and localities in Sharkia province, Egypt **(Table 3).** Such results agree with the earlier findings of the previous surveys conducted in Egypt. *Zaki and El-Sanousi (1994)* reported an incidence of 70% of CIAV antibodies in serum samples collected from broiler breeder, layer, and day old broiler flocks. *Amin et al., (1998)* stated that CIAV antibodies were detected in 97.4% of serum samples collected from 21 native and foreign grandparent, parent, and broiler flocks in 8 provinces. *Islam, (2003)* reported that seroprevalence was 74.6% and 67.3% in commercial broiler and broiler breeder flocks respectively in Sharkia province. *Hegazy et al., (2010)* confirmed the widespread nature of the virus in Sharkia province as the seroprevalence was 81.67% in commercial layer flocks and 87.78% in commercial broiler flocks. Our findings also go with that reported in other countries. Seroprevalence was 85.7% in commercial layer flocks in Afyon region, Turkey *(Kuyucuoglu et al., 2003)*; 86% in commercial broiler flocks in Nigeria *(Owoade et al., 2004)*; 87.7% in commercial broiler flocks in Shahrekord, Iran *(Mahzounieh et al., 2005)*; 82.61% in commercial broiler flocks in Northern Jordan *(Dergham, 2006)*; and 67.3% in commercial layer flocks in Khartoum state, Sudan *(Ballal et al., 2005)*.

Presence of anti-CIAV antibodies in tested flock's sera indicates that chickens may be vertically or horizontally infected or even acquired the antibodies passively from their breeders via yolk. The passively acquired antibodies are unlikely because all tested commercial layer and broiler flock's sera were collected after the age of 3 weeks, the time required for maternal antibodies to decay as mentioned by *McNulty et al., (1988)*. The presence of CIAV antibodies in tested flock's sera with no history of clinical signs or lesions suggestive to CIAV infection or vaccination against the virus certainly indicates that the source of anti-CIAV antibodies detected in tested sera is the horizontally acquired CIAV infection through direct and indirect contact with virus-contaminated dust, water or feed with feces specially that the virus shows extreme physical and chemical resistance to inactivation and so persists for long in poultry houses *(Yuasa et al., 1979)*. Although subclinical CIAV infection does not produce clinical symptoms of the disease, it is immunosuppressive *(McNulty 1991; McConnell et al., 1993)*.

Avian mycotoxicosis is considered as one of the most important problems in poultry industry in Egypt and elsewhere. It causes severe losses not only in form of performance reduction, but also as an immunosuppressive agent aggravating the bird susceptibility to diseases and magnifying mortalities *(Smith and Ross, 1991)*. It reduces the amount of antibodies following infection or vaccination, and reduces the activity of phagocytic cells *(Girish and Smith, 2008)*. Although all rations fed to the tested flocks had an antimycotoxin agent as feed additives, our results revealed the presence of aflatoxin & ochratoxin residues in rations using HPLC analysis **(Table 4).** Presence of mycotoxins in poultry rations in levels exceed the permissible levels (5ppb for aflatoxin and 20ppb for ochratoxin) impairs all production parameters and causes immunosuppression *(Smith and Ross, 1991)*. Chronic exposure to low levels of toxins -below observable overt toxicity- in rations is also likely to alter normal immune functions *(Smith and Ross, 1991; Verma et al., 2004)*.

This study suggests that the poor immune response after vaccination against AIV, manifested by non protective HI and ELISA titers, indicates immunosuppression and can be attributed to subclinical form of CIAV infection and chronic exposure to low dietary levels of aflatoxins and

ochratoxins. The wide distribution of subclinical form of chicken infectious anemia virus (CIAV) among both commercial layer and broiler flocks in Sharkia province, Egypt was proved using ELISA. Both clinical and subclinical forms of CIAV infection have destructive effect on lymphoid organs leading to immunosuppression and subsequently vaccination failure, complications with other pathogens, and great economic losses. The great need for breeders immunization and their monitoring for the presence of CIAV antibodies during rearing period is now clear to avoid vertical transmission of the virus and achieve protection of the offspring by maternal anti-CIAV antibodies. Using of high quality grads feeds and feedstuff beside detoxification processes of contaminated feeds and feedstuff may be the only solution to counteract the adverse effect of mycotoxicosis.

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