Comparative Studies on the Effect of Aflatoxins Types on the Immunization of One-Day-Old Broiler Chicks Simultaneously Vaccinated Against Newcastle Disease and Infectious Bronchitis Disease

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Abstract: This study was done to investigate the effect of the ingestion of contaminated feed with Aflatoxins types which produced with different types of fungus on the immunoresponse of one-day old broiler chicks to attenuated live virus vaccines for Newcastle disease (ND) and infectious bronchitis disease (IB) to non Aflatoxins treated groups.Concurrent exposure of chickens to 400 parts per billion (ppb) aflatoxin previously prepared from *Aspergillus parasiticus*, NRRL 2899, as a potent aflatoxigenic strain, and to 10 parts per million (ppm) Aflatoxins previously prepared from *Aspergillus flavus* NRRL 3357 as they were the most common types of fungus can produce Aflatoxins in feed and vaccination against ND, IB resulted in lack of adequate protection against subsequent experimental challenge, as assessed by antibody responses compared to chickens fed aflatoxin free ration which determined by the ELISA test. The performance parameters include food consumption, body weight, food utilization, mortality and liver pathology. **Conclusions:** The mortalities were higher in chickens fed 400 ppb of Aflatoxins from *Aspergillus parasiticus* than in the chickens fed on 10 ppm aflatoxin from *Aspergillus flavus* during the challenge test against NDV and IBV as the low levels of protective antibodies due to their immunosuppressions effect.
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1. Introduction

Vaccinal failure considered as a problem affecting broiler chicken flocks and has multifactorial causes either infectious or non infectious causes, and mycotoxins is one of the major non infectious causes which can affect on rate of gain and feed efficiency for raising healthy and profitable broiler chicken flocks, also affect seriously on the immune response of vaccination against the major infectious diseases in poultry production such as Newcastle (ND) infectious bronchitis (IB) and infectious bursal disease (IBD) as vaccination against these viral diseases were the vital to safeguard against these diseases ***(Allan et al., 1978; McFerrin & McCracken, 1988; Lukert & Saif, 1991)***. Aflatoxins (AF), a group of closely related, extremely toxic chemicals, are produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus* and can occur as natural contaminants of poultry feeds ***Edds and Bortell, (1983).*** Aflatoxins were responsible for "turkey X disease," which caused high mortality in turkey poults in England in 1960. Since then, the toxicity of AF to poultry has been well documented, as indicated by ***Huff et al. (1988).*** Aflatoxins is a type of mycotoxins as a toxic product of fungal growth produced primarily by the mold, Aspergillus flavus in cereal grains, particularly rice and corn in which its spores germinate during storage. Aflatoxins contamination of feed stuffs and prepared feeds is widespread ***(Bryden et al., 1980; Buckle, 1983; Jelinek et al., 1989; Hegazi et al.,1991).*** Our Studies evaluate the effect of Aflatoxins types as immunosuppressive due to its ingestion in feed has which resulted in decreased immunity in vaccinated birds ***(Campbell et al., 1988; Gush et al., 1990; Hegazi et al., 1991; Mohiudin, 1993; Azzam & Gabal, 1997). A***lso its economical effects on the mortality rates, body gains, feed conversions rate and increase the condemnation rates of both contaminated ration and carcasses of chickens. These levels of aflatoxicosis produced signs and lesions as well as a significant decrease in weight gain and feed conversion during 5 weeks. In addition, microscopic lesions, indicative of aflatoxicosis, were evident and significant decreases in neither humoral immunity nor the development of the acquired immunity to Newcastle disease or Infectious bronchitis.

2. Material and Methods

1- Experimental Chicks:

A total of 210 One-day-old commercial broiler Hubbard chicks were used (El Arabia Company for poultry production, Cairo). The chickens were fed a commercial corn-soybean meal starter ration formulated to meet or exceed the recommended nutrient requirements ***National Research Council,***

(***1984)*** and housed in heated starter batteries under continuous fluorescent lighting. Experimental diets and water were provided for *ad libitum* consumption. Individual feed intake and BW were recorded weekly. These chicks were derived from breeders vaccinated with both live and inactivated IB and NDV vaccines. Individually weighed, wing banded.

2-Aflatoxins

Two groups of Aflatoxin were prepared according to the type of fungus producing it:

1-Aflatoxin was locally prepared by the kindly help of (National Reach center Laboratory, Giza) as produced via fermentation of rice by Aspergillus parasiticus NRRL 2999 as described by ***Shotwell et al. (1966)*** and modified by ***West et al. (1973).*** Fermented rice was autoclaved and ground and the AF content measured by spectrophotometric analysis ***Nabney and Nesbitt, 1965; Wiseman et al., (1967).*** Of the total AF content in the rice powder, 79% was AFB1, 16% was AFG1, 4% was AFB2, and 1% was AFG2. The rice powder was incorporated into the basal diet and confirmed by HPLC to provide the desired level of 4 mg AF/kg (400 parts/billion ppb)/chicken/day. Aflatoxin concentration of diets was based on AFB1

2-Another Aflatoxin prepared locally in our laboratory by growing Aspergillus flavus NRRL 3357 was grown on enriched long-grain rice for 7-10 days to produce aflatoxin B1 (AFB1). The quantity of AFB1 in moldy rice was determined by thin-layer chromatography using ultraviolet light. Used as dried moldy rice powder was fed in unmedicated feed by (AFB1 level 10 ppm).

-The two types of aflatoxin feeding were continued for 2 months during the experiment

3- Vaccines:

1- ***AVINEW***(Merial, France) Live vaccine against Newcastle disease VG/VA Strain – Freeze-dried pellet.

Composition:

The Vaccinal strain is the VG/GA strain of Newcastle disease virus. Each dose contains at least 15 PD90 (protective dose 90%)

Doses:

Use within 2 hours after reconstitution with physiological saline or special diluent via ocular route individually by dose of 10^3 EID 50

AVINEW a Lentogenic virus isolated by Gilson and Villages from digestive system (intestine) of turkey ***(Glisson et al., 1990;Mayo, 2002; Nunes, et al., 2002;).***

2- **Gallimune ND:** Inactivated vaccine against Newcastle disease.

Composition:

The vaccine contains inactivated Newcastle disease virus, a preservative and an oil excipient .As a booster for the respective vaccination.

Dosage*:*

0.3 ml dose per chicken

3-**Nobilis® IB H120** (Merck AH the Netherlands) **is** a live vaccine against Infectious Bronchitis serotype Massachusetts (strain H120) in poultry. H120 IBV live vaccine was used.

Composition:

Active components per dose: Live IB strain H120: >= 3.0 log10 EID50 as freeze-dried pellet.

4-**Nobilis® Ma5 + Clone 30** (Merck AH the Netherlands) is a live vaccine against Infectious Bronchitis serotype Massachusetts (strain Ma5) and Newcastle Disease (strain Clone 30) in chickens.

Composition*:*

Active components per dose:

- Live I.B. strain Ma5: >= 3.0 $\log^{10}$ EID_{50}
- Live ND strain Clone 30: >= 6.0 $\log^{10}$ EID_{50}

AS freeze-dried pellet *intraocular instillation procedure:* Dissolve the vaccine in physiological saline solution (usually 30 ml per 1000 doses) and administer by means of a standardised dropper. One drop should be applied from a height of a few centimetres into one eye. The handler should ensure that the eye drop is inhaled by the bird and then free the chicken.

Vaccination via injection:

Shake well prior to use Subcutaneous and/or intramuscular route

Procedure:

The chicks groups were vaccinated at one day old by ND, IB and combined ND + IB Live vaccines through ocular instillation. The all chicks groups were revaccinated against ND via ocular instillation using ND live vaccine (VG/GA strain- Avinew), at $15^{th,}$ 30^{th} days of age. The vaccine was diluted to give each bird approximately a dose of 10^3 EID $_{50}$ and finally vaccinated at 40^{th}, 55^{th} days of age by inactivated ND vaccine.

4-Embryonated chicken eggs

SPF E.C.E obtained from Nile SPF (Koom Oshiem, Fayoum, Egypt) were used for titration of the vaccines strains

5-Vaccine titration:

The used vaccines were titrated in SPF embryonated chicken eggs according to ***(Villegas and Purchase,* 1989*),*** the titer was expressed as 50% embryo infective dose(EID_{50})/ml and it was calculated as ***Reed and Munch (1938).***

6-Challenge virus strains

NDV, IBV strains, obtained from the National Reachers center Laboratories, Giza, Egypt. Challenge viruses were given by eye drop bilaterally applying (0.2 ml) on each eye of $10^{3.5}$ median egg infectious

dose (EID_{50})/ml NDV and 10^4 EID^{50} of IBV Challenged groups were isolated in cages in separate rooms and Control, nonchallenged groups all groups were observed for 14 days post challenge. Symptoms, mortality and grossly visible lesions at necropsy were recorded.

7-Serum samples

Blood samples were collected every two weeks from individual chickens in all groups, from wing vein in clean dry, sterile Wassermann tubes. The tubes containing blood samples were stroppered and left in horizontal position for an hour at room temperature and then left for another hour at 4°C then centrifuged at 3000 r.p.m. for 15 minutes. Sera were separated, inactivated at 56°C for 30 minutes in a water bath and frozen at - 20°C until tested at the end of the experiment to determining the antibody titres of NDV, IBV by ELISA test.

5-ELISA Kits:

NDV and IBV-ELISA Kits were obtained from Kikegaard and Perry laboratories (Kpl), U.S.A.

6- ELISA test procedures:

ELISA test was carried out according to manufactural instructions as while

Calculation of ELISA titers:

- Negative control mean (NCx) = well $\frac{A1+ well A2}{2}$ = NCx

- Positive control mean (PCx) = $\frac{well A3+ well A4}{2}$ = PCx

- S/P ratio = $\frac{sample mean - NCx}{PCx - NCx}$ = S/P

-Titer- Relates S/P at a 1:500 dilution to an endpoint titer: Log_{10} titer=1.09(log_{10} S/P)

8-Statistical analysis:

Statistical Analysis of variance **(ANOVA)** test was used to estimate differences among treatments according to ***(Steel and Torrie, 1960).*** Correlation and linear regression analysis were also performed using Microsoft excel program.

9- Performance parameters:

Performance parameters for broiler chicks including average weekly mortality rate, body weight gain /gm., cumulative feed intake/gm (CFI/gm) and feed conversion rate (FCR) were used and calculated according to ***Sainsbury (1984).***

10-Expermint procedure:

230 One-day-old commercial broiler Hubbard chicks were divided into four groups **A,B,C,D and E** ,the groups **A,B,C** consists of 60 one day old chick and the group **D** and **E** have 20 one day old chick for each **D** kept as vaccinated ,none Aflatoxins fed and challenged while group **E** kept as non vaccinated ,free aflatoxin feeding control (control –ve)

-The group **A** (vaccinated intra ocular at one day old by ***AVINEW***for **ND)**

- The group **B** (vaccinated intra ocular at one day old by **Nobilis IB H120**)

- The group **C** (vaccinated intra ocular at one day old by **Nobilis® Ma5 + Clone 30**)

-The groups **A, B, C** were revaccinated with *AVINEW* for ND at 15th, 30th days of age the revaccinate by the inactivated vaccine against ND at 40th and 55th days of age

- The groups **A, B, C** were divided into sub groups (A1, A2), (B1, B2) and (C1, C2) the sub group A,B of 30 sub group C was 40 one day old vaccinated chicks as previously stated.

-The sub groups (A1, B1, and C1) feed on ration contain Aflatoxin produced by Aspergillus parasiticus, NRRL 2899, a potent aflatoxigenic strain, by a dose 4 mg AF/kg (400 parts/billion ppb)/chicken/day for 8weeks

-While the other sub groups (A2, B2, and C2) feed on ration contain Aflatoxin produced by Aspergillus flavus NRRL 3357 by a dose level 10 ppm of AFB1/chicken/day for 8weeks

-Challenging for each group by NDV (28th day old), IBV (40th day old) Challenge viruses strains were given at 28th and 40th days of age respectively for each virus strain by eye drop bilaterally applying (0.2 ml) on each eye of $10^{3.5}$ median egg infectious dose (EID_{50})/ml NDV and 10^4 EID^{50} of IBV Challenged groups were isolated in cages in separate rooms and Control, nonchallenged groups all groups were observed for 14 days post challenge. Symptoms, mortality and grossly visible lesions at necropsy were recorded.

3. Results and Disscusion

In this study, we directed our work to investigate the comparative effect of the ingestion of contaminated feed with Aflatoxins types which produced with different types of fungus on the immunoresponse of one-day old broiler chicks to the most popularly attenuated live virus vaccines for Newcastle disease (ND) and infectious bronchitis disease (IB) to non Aflatoxins treated groups in the presence of maternal antibodies in chicken flocks, experiment design shown in table (1), Vaccination of 1- day old broiler chicks which possess natural maternal antibodies show pronounced immunity between 3 and 4 weeks of age, the ability of mothers to transmit antibodies to their offspring was documented in both mammals and birds over100 years *ago* ***(Giambrone and Ronald, ,1986); Jennifer et al., (2003) and Hamal et al., 2006)*** , maternal antibodies is protective and during the vaccination the maternal antibodies neutralize the vaccine antigen rendering the vaccine in effective ,also the age of chicks at vaccination and the level of maternal antibody greatly influence immune response of broiler chickens to the vicinal antigen

(Awang et al.,1992). Data on the effects of aflatoxin on antibody titres are shown in Tables 3 to 5. The titres were markedly higher in the vaccinated, non-aflatoxin exposed groups than in those exposed to aflatoxin. They were also considerably higher than the values of the negative controls (data not shown) provided with each ELISA kit. The statistical analysis of the ELISA data showed that the effect of aflatoxin on the titres was highly significant. No significant differences were observed between the different groups in relation to whether the vaccines were administered singly or in combination. The titres in the non-vaccinated groups seemed to be correlated to maternal immunity. Mortality was higher following challenge in the aflatoxin exposed groups especially which exposed to NRRL2899 (Aspergillus parasiticus) 2oo ppb compared to the other group which exposed to NRRL3357 (Aspergillus flavus) 10 ppm and the non aflatoxin exposed groups **(Table 2,)(Fig. 1).** There was mortality in the IBV-challenged groups at an older age. Chickens which died following IB challenge in the aflatoxin exposed and vaccinated groups showed symptoms and postmortem findings similar to those found in the non vaccinated chickens, including tracheitis with or without catarrhal discharge, caseous plugs in the lower trachea or bronchi, pneumonia and varying degrees of air sacculitis. Those challenged with NDV showed lesions in Proventriculus, caecae and small intestines with and without septicaemic findings.

We found that, the ingestion of aflatoxin contaminated feed significantly lowered antibody titres in chickens immunized against ND and IB compared to non aflatoxin treated groups. The immunosuppressive effect of aflatoxin has been related to its direct inhibition of protein synthesis, including those with specific functions such as immunoglobulins IgG, IgA, inhibition of migration of macrophages, interferance with the haemolytic activity of complement, reduction in the number of lymphocytes through its toxic effect on the Bursa of Fabricius as in histopathological section of bursa found lymphocytic depilation and bursa vaccuolation ***(M. Denli et al., 2009)*****(plate 1)** and impairment of cytokines formation by lymphocytes ***(Richard et al., 1974; Pier et al., 1979; Campbell et al., 1988; Azzam & Gabal, 1997).*** Although mortality from IB field outbreaks usually occurs in birds up to 5 weeks of age, the mortality in our study in older chickens may have been attributed to the virulence of the challenge strain used and/or the challenge dose. Some IBV strains have been characterized as nephropathic ***(King & Cavanagh, 1991).*** Aflatoxin is also a potent nephrotoxin and the continued exposure to aflatoxin resulted in tremendous kidney damage and thus made it easier for IBV to kill the birds. The suppression of the immunoresponse was observed in birds vaccinated with single or combined vaccines. However, no significant effect or adverse interaction on the titres was observed between the groups which received a single versus combined vaccination ***(Hanson et al., 1956; Thornton & Muskett, 1975).*** Major infectious diseases of poultry have been controlled by immunization and effective management practices. Lack of adequate protection and interference with immunity of birds seem to have important roles in such cases. Aflatoxin is an immunosuppressant of widespread nature in feed and feed raw materials, and exposure of poultry to subclinical doses of aflatoxin has been shown to cause infection, even among immunized birds in field situations. Outbreaks of fowl cholera and IBD have been reported in vaccinated flocks associated with ingestion of aflatoxin contaminated feed ***(Hegazi et al., 1991; Anjum, 1994).*** The widespread distribution of both Aspergillus parasiticus and flavus were the main fungal species which produces aflatoxin in feed and raw feed materials, suggests that aflatoxin contamination must be seriously considered in the poultry industry. Although several measures have been introduced to alleviate contamination problems, costs are still a major barrier for their general use ***(Gabal, 1987;Jindal et al., 1993;Devegowda et al., 1994; Hirano et al., 1994;).*** Until more cost efficient solutions are found to prevent aflatoxin from reaching the food chain, regular and vigorous quality controls of feed are required to safeguard poultry.

Table (1) *Experimental design*

groups		Types of aflatoxin fed	Type of vaccine and age of vaccination(days)				
			1 day	15th	30th	40th	55th
A	A_1	NRRL2899(Aspergillus parasiticus)4oo ppb	Live attenuated ND vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Inactivated ND vaccine
	A_2	NRRL3357(Aspergillus flavus)10 ppm					
B	B_1	NRRL2899(Aspergillus parasiticus)4oo ppb	Live attenuated IB vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Inactivated ND vaccine
	B_2	NRRL3357(Aspergillus flavus)10 ppm					
C	C_1	NRRL2899(Aspergillus parasiticus)4oo ppb	Live attenuated ND+IB vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Inactivated ND vaccine
	C_2	NRRL3357(Aspergillus flavus)10 ppm					
D		none	Live attenuated ND+IB vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Live attenuated ND vaccine	Inactivated ND vaccine
E		none	none	none	none	none	none

Table (2) Mortality % in challenged, vaccinated and aflatoxin exposed groups

Groups		Types of aflatoxin fed	Number of chicks in each group	Type of challenged virus strain	Total Number of dead chicks	Mortality rate %
A	A_1	NRRL2899(Aspergillus parasiticus)4oo ppb	15	NDV	11	73.3%
	A_2	NRRL3357(Aspergillus flavus)10 ppm	15	NDV	6	40%
B	B_1	NRRL2899(Aspergillus parasiticus)4oo ppb	15	IBV	8	53.3%
	B_2	NRRL3357(Aspergillus flavus)10 ppm	15	IBV	5	33.3%
C	C_1	NRRL2899(Aspergillus parasiticus)4oo ppb	10^a	NDV	9	90%
			10^b	IBV	5	50%
	C_2	NRRL3357(Aspergillus flavus)10 ppm	10^c	NDV	6	60%
			10^d	IBV	4	40%
D	D_1	none	10	NDV	1	10%
	D_2	none	10	IBV	-	0%
E		none	20	-	-	-

a: number of chicks in groupC_1 treated with NRRL 2899 and challenged with NDV ,b: number of chicks in groupC_1 treated with NRRL2899 andchallenged with IBV ,c: number of chicks in groupC_1 treated with NRRL 3357 and challenged with NDV, d: number of chicks in groupC_1 treated with NRRL 3357 and challenged with IBV

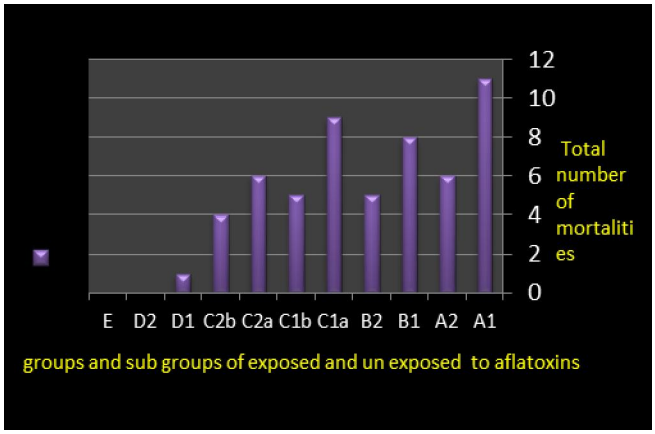

Fig (1) Total number of mortalities in groups and subgroups of exposed and unexposed to aflatoxins in challenged vaccinated chicks.

Table (3): Mean ELISA antibodies titers of Aflatoxins exposed and unexposed chickens vaccinated with live attenuated NDV (Avinew) and challenged with NDV strain

Age(days)	Vaccinated ,challenged non exposed to Aflatoxins	Vaccinated ,challenged exposed to Aflatoxins	
	D_1	A_1	A_2
0	$9634^a \pm 436$	$9845^b \pm 324$	$9845^b \pm 324$
15	$10956^a \pm 423$	$4162^b \pm 221$	$6342^b \pm 312$
30	$8432^a \pm 342$	$2186^b \pm 264$	$4321^b \pm 231$
45	$7013^a \pm 278$	$1218^b \pm 79$	$3229^b \pm 145$
60	$6281^a \pm 207$	$820^b \pm 17$	$1455^b \pm 79$

S.E: standard error superscripts letter (a &b)are significantly different ($P< 0.05$).

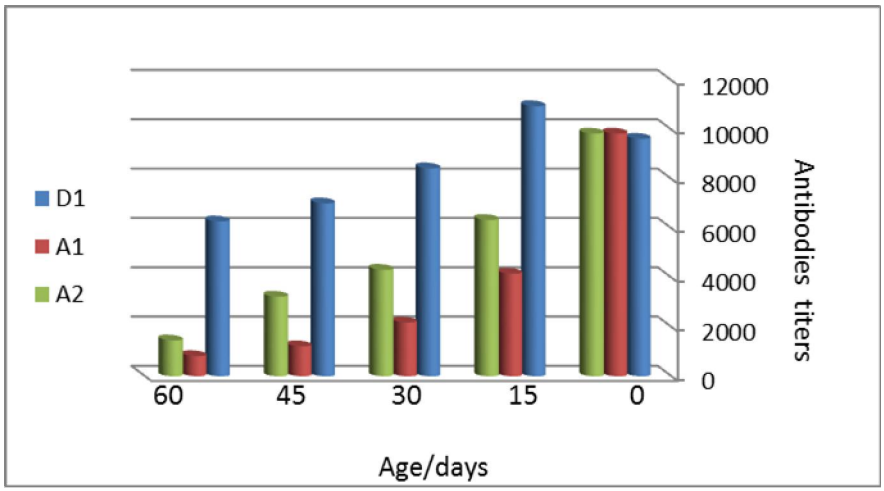

Fig (2) Mean ELISA antibodies titers of Aflatoxins exposed and unexposed chickens vaccinated with live attenuated NDV (Avinew) and challenged with NDV strain

Table (4) Mean ELISA antibodies titers of Aflatoxins exposed and unexposed chickens vaccinated with live attenuated IB H120 vaccine and challenged with IBV strain

Age(days)	Vaccinated ,challenged non exposed to Aflatoxins	Vaccinated ,challenged exposed to Aflatoxins	
	D_2	B_1	B_2
0	8394^{a} ±454	8485^{b} ±366	8485^{b} ±366
15	9956^{a} ±478	3682^{b} ±271	5740^{b} ±279
30	8136^{a} ±316	2456^{b} ±214	4870^{b} ±298
45	6824^{a} ±278	988^{b} ±60	3406^{b} ±147
60	5791^{a} ±237	805^{b} ±13	985^{b} ±76

S.E: standard error superscripts letter (a &b)are significantly different ($P< 0.05$).

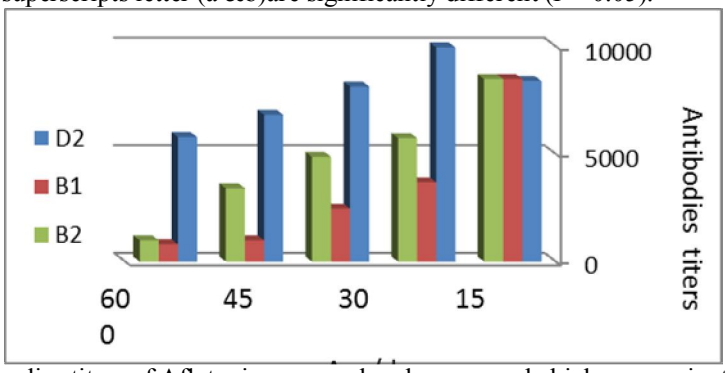

Fig.(3) Mean ELISA antibodies titers of Aflatoxins exposed and unexposed chickens vaccinated with live attenuated IB H120 vaccine and challenged with IBV strain.

Table (5) Mean ELISA antibodies titers of Aflatoxins exposed and unexposed chickens vaccinated with live attenuated Ma5 + Clone 30vaccine and challenged with IBV and NDV strains

Age(days)	Vaccinated ,challenged non exposed to Aflatoxins		Vaccinated ,challenged exposed to Aflatoxins			
	D		C_1		C_2	
	D_1	D_2	C_{1a}	C_{1b}	C_{2a}	C_{2b}
0	9634^{a} ±436	8394^{a} ±454	9485^{b} ±382	9485^{b} ±382	9485^{b} ±382	9485^{b} ±382
15	10956^{a} ±423	9956^{a} ±478	4972^{b} ±312	5492^{b} ±357	6321^{b} ±322	5234^{b} ±362
30	8432^{a} ±342	8136^{a} ±316	3480^{b} ±307	2750^{b} ±287	3456^{b} ±212	4283^{b} ±297
45	7013^{a} ±278	6824^{a} ±278	2187^{b} ±169	1987^{b} ±194	1769^{b} ±143	3861^{b} ±310
60	6281^{a} ±207	5791^{a} ±237	1678^{b} ±93	$989\pm^{b}$ 63	753^{b} ±28	1674^{b} ±69

S.E: standard error superscripts letter (a &b)are significantly different ($P< 0.05$).

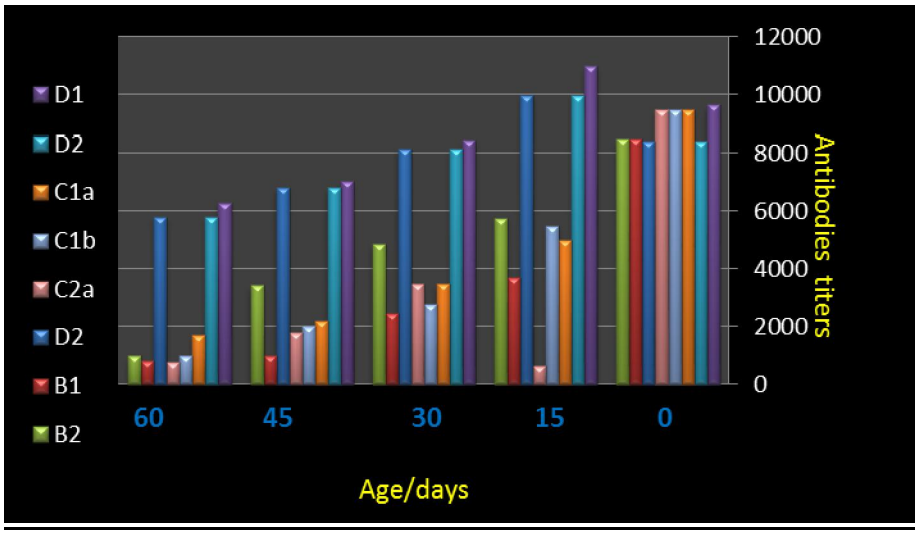

Fig (4):Mean ELISA antibodies titers of Aflatoxins exposed and unexposed chickens vaccinated with live attenuated Ma5 + Clone 30vaccine and challenged with IBV and NDV strains

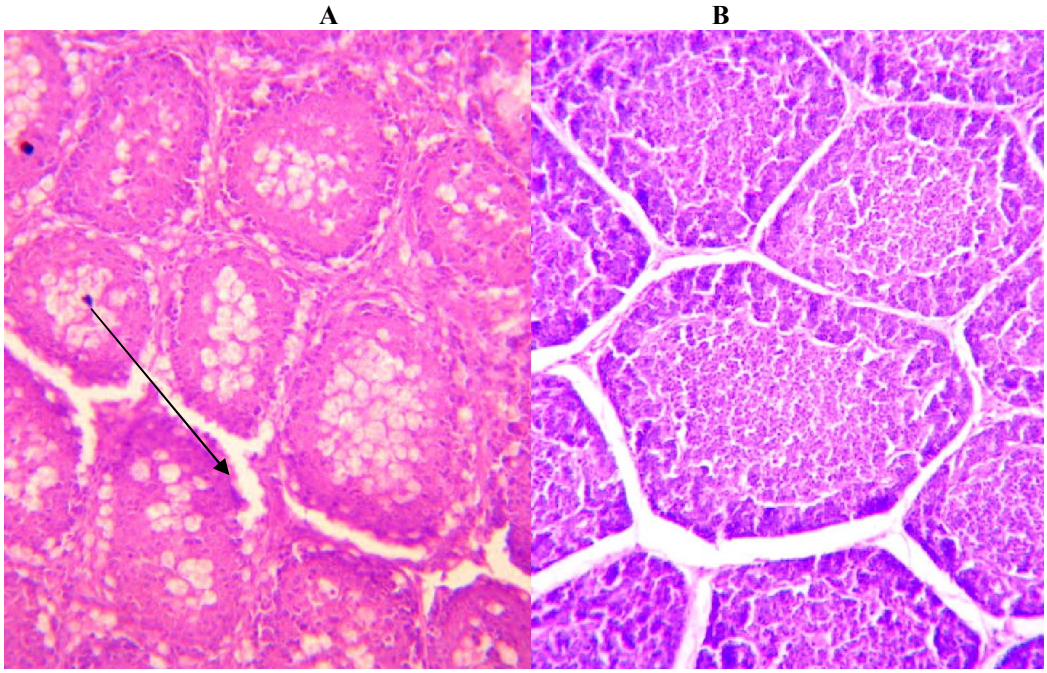

Plate (1): A, Bursal sections of bursa at 35 days age showing: Severe lymphocytic depletion and necrosis (arrow) and medulla of lymphoid follicles showed vacuolated reticular cells cyst formation (Lesion score: 5) X200.stained with H&E; **B**, normal control bursa

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