

# Research Journal of Pharmaceutical, Biological and Chemical Sciences

## Semi Field Study for Evaluation of Broiler Immune Response to Used Newcastle And Avian Influenza Vaccines.

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### ABSTRACT

The effect of tilmicosin and colistin sulphate on immune response of broiler chickens against live and inactivated Newcastle disease (ND) as well as inactivated Avian influenza (AI) vaccine in presence or absence of prebiotic was studied. A total number of 225 broilers Ross chicks were divided into 9 equal groups; 25 chicks in each. Groups 2-8 and group 9 were vaccinated with live + combined inactivated and live+ single inactivated vaccine; respectively. Group 1 and 8-9 were kept as control negative (non treated non vaccinated) and non treated vaccinated; respectively. Chicken groups 2,3,4,5,6 and 7 were treated with tilmicosin + colistin, tilmicosin, tilmicosin and prebiotic, colistin, colistin + prebiotic and prebiotic; respectively. Blood samples were collected at 0, 10, 20 and 30 days of age for sera and subjected to haemagglutination inhibition (HI) test. Bursa, spleen and liver were collected at the 30<sup>th</sup> day of life for histopathological examination. The control negative group showed decline maternal HI titres from  $3.13 \pm 1.95$  and  $3.67 \pm 0.89$  at 0 day to reach  $1.33 \pm 1.18$  and  $0.73 \pm 1.10$  at 30 days for ND and AI; respectively. Results of HI test against ND by the 30<sup>th</sup> day of life (12 days post last vaccination). revealed that highest HI antibody titers was  $5.51 \pm 1.62$  followed by  $5.07 \pm 1.53$ ;  $4.95 \pm 1.46$ ;  $4.87 \pm 1.13$ ;  $4.74 \pm 1.03$ ;  $4.73 \pm 1.28$ ;  $4.53 \pm 1.38$  and  $4.47 \pm 1.41$  group 6, 9, 7, 4, 5, 2, 8 and 3 (tilmicosin group); respectively. Results of HI test against AI H5N1 vaccine at the 30<sup>th</sup> day of life revealed that highest titer was  $4.88 \pm 1.19$  followed by  $4.82 \pm 0.91$ ,  $4.81 \pm 1.44$ ,  $4.69 \pm 0.89$ ,  $4.67 \pm 0.90$ ,  $4.60 \pm 1.24$ ,  $4.55 \pm 1.10$  and  $4.35 \pm 0.82$  in gr 6 (colistin+ prebiotic) which, gr 7 (prebiotic only), gr 9 (single inactivated AI and ND), gr 5 (colistin), gr 2 (tilmicosin+colistin), gr 8 (combined inactivated ND-AI vaccine), gr 4 (tilmicosin+ prebiotic) and gr 3 (tilmicosin); respectively. Regarding histopathological findings revealed that control negative group (1) did not show any pathological changes during whole of the experiment, while bursa of control positive vaccinated group showed moderate hyper plastic activity of the lymphoid follicle while spleen showing severe hyperplastic activity of the lymphoid follicle, Group (5) received colistin showing vacuolar degeneration in the hepatocytes by the end of the experiment, while bursa of Fabricus of group (7) received prebiotic mixture showing severe hyperplastic activity of the lymphoid follicle and the epithelial lining showing hyper plastic activity forming finger like projection. Control positive group vaccinated non treated liver showing severe congestion of the central vein and sinusoids, vacuolar degeneration of the cytoplasm in the hepatocyte with disorganization of the hepatic cord. It could be concluded that the use of antibiotics in poultry industries can induce either bad or positive effect on chicken humoral immune response expressed by HI antibodies against inactivated poultry vaccines this depends on its class which belonged on, moreover prebiotics was found to have a great value in improving or restore activity of immune response and performance of vital organs against effects of antibiotics. Under the condition of our work it was noticed that the usage of single inactivated vaccines against two viruses resulting in better immune response than combined inactivated vaccine this results needs further investigation.

**Keywords:** broiler chicken, AI+ND inactivated vaccine, HI test, Antibiotic, prebiotic, histopathology.

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## INTRODUCTION

Antibiotic antibacterial medications still widely used in poultry industry in several indications including therapeutic treatment, prevention or as traditional growth promoters [1] and [2], however using of such antibiotics at time of vaccination is not well established yet and few data available in such indication. Macroloids are widely used nowadays such as tilmicosin which is bacteriostatic macrolide antibiotic synthesized from tylosin for veterinary use only has antibacterial spectrum mainly against atypical microorganism (*Mycoplasma* spp., *Pasteurella* spp. and many Gram- positive microorganisms [3], also other researchers reported that tilmicosin macroloid antibiotic has negative effect on humeral immune response and resulted in decrease in antibody titers, this decrease in titer does not affect the protection titer of vaccinated birds, on the other hand cellular immune response was improved of vaccinated chickens treated with such tilmicosin antibiotics [4] as it concentrated in phagocytes due to its lipophilic properties [5] resulting in increase in lysozyme activities. concerning colistine sulphate is one of polymyxins; cationic lipopeptides. [6], used in treatment of resistant strains gram negative microorganisms in poultry industry [7] unfortunately continues use of this antibiotic is a direct cause of toxicity [8] including hepatotoxicity and or nephrotoxicity [9] on the other hand use of such polymyxins antibiotic found to improves humoral immune response against viral respiratory diseases in poultry [10], moreover the use of such antibiotic as antibacterial preventing vaccines contamination during production of poultry vaccines and was found it stimulating of more humoral immune response [11]. Viral respiratory disease of poultry cause severe economic losses specially AI and ND, which could be prevented by proper vaccination program using live and inactivated vaccine against ND or inactivated vaccine only in AI virus. Many medicinal extract are prebiotics improves humoral immune response, such as garlic oil [12], ginger powder [13], vitamin E [14] and vitamin A [15], this products may presented as patent preparation together in market which induce synergistic actions for better results.

Our study was designated to investigate the effect of commercial prebiotics mixture (Gar Vit Pro<sup>®</sup>) when used as feed additive to improve or restore the suppressive effect of commonly used antibiotics on humeral immune response as measured by HI- test against ND and AI vaccination as used in Egyptian field with especial attention on comparing results found by either combined or single AI and ND inactivated vaccine.

## MATERIAL AND METHODS

### Experimental Chicks:

A total number of 225 broilers Ross chicks were divided into 9 equal groups; 25 chicks in each. Chicken groups were kept in separate clean disinfected rooms on deep litter.

### Ration

Commercial starter and grower broiler chicken ration were provided till 21 and 32 days of age, respectively. The chicks were feed on prepared ration according to the Hubbard broiler management manual and NRC (1984) requirement for broiler. All housed chickens were given ration and water ad libitum. The ration contained the coccidiostate.

### Vaccinal Strains

- Hitchiner B1 vaccine: contains lentogenic Newcastle strains – Hipra lab. – Spain - Batch NO. 388V-3.
- La Sota vaccine: lentogenic Newcastle strains – IZO S.P.A. – Italy Batch no. 0722 F.
- Inactivated ND Clone 30 virus " Newcavac vaccine" - Intervet UK Ltd- Batch no. S257A01.
- Inactivated oil VOLVAC<sup>®</sup> AI H5N2 Inactivated oil emulsion vaccine - Boehringer Ingelheim vetmedica S. A. De. C. V., Mexico. recommended dose according company instructions was 0.5 ml, used subcutaneously in neck region.
- VOLVAC<sup>®</sup> AI – ND: inactivated oil emulsified AI virus Type A, subtype H5N2,A/CHICKEN/MEXICO/232/94/CPA strain and ND. Minimum titer for AI and ND is  $10^{7.6}$  EID<sub>50</sub>/dose and  $10^{8.2}$  EID<sub>50</sub>/dose; respectively.

**Prebiotic:**

**Gar Vit Pro®** : prebiotic contains Vit A, Vit. E, Garlic oil, Ginger, Celandine, Horse tail and Willow bark – Batch No. 141091409404/94. – Germany.

**Antibiotics:**

**Colistin sulphate 6 MIU**: each gm contains 6000.000 IU colistine sulphate. Lot No. 150415 . Jordan Vet. and Agr. Med. Ind. Co – Amman – Jordan.

**TILMOSOL 25%** : each 100ml contains tilmicodin phosphate 28gm equivalent to 25 gm tilmicodin base – produced by Arabcomed company – registration number 2011/3343.Egypt.

**Vaccination time and application methods:**

Live vaccines were administered via eye drops instillation while inactivated vaccine against avian influenza (AI-ND) disease CEVA® at 7 days old of age through subcutaneous route at the back of the neck.

**Embryonated chicken eggs (ECEs):**

Specific pathogen free (SPF) obtained from Kom Oshim, El-Fayoum, Egypt. ECEs were used for vaccine titration and propagation of virus vaccine and HI antigen. Embryonated chicken eggs were inoculated according to [16,17].

**Titration of Live vaccine:**

Live vaccinal virus was titrated according to [16] in specific pathogen free (SPF) embryonated chicken egg (ECE) at 9 days of embryo life and titer calculated by [18] viral titer was  $10^{9.25}$  and  $10^{9.07}$  EID<sub>50</sub> for Hitchiner B<sub>1</sub> and La Sota vaccine; respectively.

**Samples:**

Fifteen blood samples for sea were collected for HI-test at 0, 10, 20 and 30 days of life. Bursa, spleen and liver samples were collected for histopathological examination at the 30 day of life.

**Hemagglutination (HA) antigens:**

AI HA antigen was kindly obtained from Animal Health Research Institute, Dokki, Giza ; while ND HA antigens were prepared by propagating of La Sota life vaccine was on ECE and used as HH antigen . Both used antigens were titrated to 4 HA units using HA test according to WHO [19].

**Haemagglutination inhibition (HI) test:**

Serum samples were tested to evaluate the antibodies titer against ND and AI, using the standard HI method The test was carried out according to the standard procedure described by *Majiyagbe and Hitchner* [20] The end point were given titer reference numbers according to scheme described by *Kaleta and Siegmann* [21].

**Histopathological Studies:**

Tissue specimens from liver ,bursa of fabricus and spleen of experimental birds of each group chicks were fixed in 10% neutral formalin solution and the specimens were routinely processed in paraffin embedding method ,sectioned and stained with Haematoxylin and Eosin (H&E) for light microscopic examination according to *Bancroft, and Gamble*, [22].

**Experimental design:**

Experimental design is shown in table (1), a total number of 225 broilers chicks were divided into 9 equal groups; 25 chicks in each. chicken group 1 kept was kept as control negative (non treated non vaccinated group). Group 2 received tilmicosin + colistin antibiotic , group 3 received tilmicosin , group 4 received tilmicosin and prebiotic, group 5 received colistin only , group 6 received colistin + prebiotic , group 7 received prebiotic only ,group 8 considered non treated vaccinated with inactivated combined vaccine and finally group 9 considered non treated vaccinated with inactivated ND and AI vaccine. Chicken groups 2-9 were vaccinated by eye drops instillation with ND live Hitchner B1 and La Sota at 7 and 18 days of age ; respectively. While inactivated was used through subcutaneous route at the back of the neck in groups 2 - 9. Group 8 received combined inactivated ND-AI vaccine without treatment and group 9 received single inactivated ND vaccine at 8 days and single AI inactivated vaccine at 8 and 10 days of age; respectively. Antibiotics used at the first 3 days ,15-17 and 26-28 days of age, while prebiotic mixture application start from the second days 2 of life till the end of experiment. Fifteen blood samples for sea were collected for HI-test at 0, 10, 20 and 30 days of life. Bursa, spleen and liver samples were collected for histopathological examination at the 30 day of life. The obtained results are shown in tables (2 and 3) fig (1-7).

**RESULTS AND DISCUSSION**

Broilers raisers use different medications during rearing period even at vaccination time without dealing with effect of this medication on immune response against used vaccines specially ND and AI vaccines. Results of main HI titers against ND virus are shown in table (2) . The control negative group showed decline maternal HI titres from  $3.13 \pm 1.95$  and  $3.67 \pm 0.89$  at 0 day to reach  $1.33 \pm 1.18$  and  $0.73 \pm 1.10$  at 30 days for ND and AI ; respectively.

By the 30<sup>th</sup> day of life results of HI test against Newcastle vaccine (12 days post last vaccination) are shown in table (2) fig (1), the highest HI antibody titers was group 6 (received colistin + prebiotics ) which was  $5.51 \pm 1.62$  followed by group 9 (received single inactivated avian influenza and newcastle inactivated vaccine ) which was  $5.07 \pm 1.53$ , followed by group 7 (received prebiotic alone) which was  $4.95 \pm 1.46$  , followed by group 4 ( received tilmicosin + prebiotic ) which was  $4.87 \pm 1.13$ , followed by group 5 ( received colistin ) which was  $4.74 \pm 1.03$  , followed by group 2 (received tilmicosin+ colistin ) which was  $4.73 \pm 1.28$ , followed by group 8 ( vaccinated with combined vaccine non treated ) which was  $4.53 \pm 1.38$  , followed by group 3 ( tilmicosin group ) which was  $4.47 \pm 1.41$  , followed by control negative group (1) which was  $1.33 \pm 1.18$

Immunity against Newcastle vaccine derived from neutralizing antibody against haemagglutinin and fusion glycoprotein [23], resulting in protective HI titer in 85% of flock protecting birds against field virus infection [24].

Results of HI test against AI H5N1 vaccine at the 30<sup>th</sup> day of life were shown in table (3) fig (2). Highest titer was gr 6 (colistin + prebiotic ) which was  $4.88 \pm 1.19$ , followed by gr 7 ( received prebiotic) which was  $4.82 \pm 0.91$ , followed by group 9 ( vaccinated with single inactivated AI and ND vaccine ) which was  $4.81 \pm 1.44$ , followed by gr 5 (colistin) which was  $4.69 \pm 0.89$ , followed by gr 2 ( tilmicosin+colistine) which was  $4.67 \pm 0.90$  ,followed by group 8 (vaccinated with combined inactivated ND-AI vaccine) which was  $4.60 \pm 1.24$ , followed by group 4 (tilmicosin+ prebiotic ) which was  $4.55 \pm 1.10$ , followed by group 3 (tilmicosin ) which was  $4.35 \pm 0.82$ , followed by gr 1 ( control negative group ) which was  $0.73 \pm 1.10$ .

Field protection against avian influenza mainly by inactivated vaccine in order to eradicate and decrease virus shedding of the disease [25], also antibody produced against AI is widely based on production of neutralizing antibody against HA epitops which considered immunogenic neutralizing epitops and antibodies created mainly protect against homologous isolates [26].

It was noticed that HI titer against inactivated combined AI-ND vaccine non treated group (gr 8) was lower compared with those received single inactivated vaccine against each disease alone (gr 9) this maybe due to type of oil adjuvant which is differ from vaccine to another that affect immune response [27], or difference in antigenic mass as it was found that efficiency of inactivated whole influenza commercial vaccine depends on antigenic mass content ,similarity between field challenge virus and vaccinal virus and to lesser extent composition of the oil emulsion [28, 29].

Results of group 1 indicate normal decaying of maternal antibody revealed no challenge during whole experimental time.

Results of measured HI humoral immune response against both ND and AI inactivated vaccines revealed that timicosin group showing lower antibody titers when compared to vaccinated non treated group, this results was matched with Khalifeh et al. [4] who reported that timicosin reduce humoral immune response against respiratory virus vaccine but had beneficial effects on the cell-mediated immune response, on the other hand prebiotic mixture improves humoral immune response when added in test groups resulting in not only HI titers but also improve immune response in timicosin test group.

Prebiotics found to improves humoral immune response cell-mediated immunity [30, 31, 32]. In our study prebiotic mixture found to improves humoral antibody titers against both Newcastle vaccination with live and inactivated vaccine and inactivated influenza vaccine, this may be due to its component including garlic oil as some researcher found that it improves humoral immune response due its content alone [33] or when present in combination with other prebiotics [34], other components in our prebiotic mixture found to improves humoral immune response such as ginger powder [13], vitamin E [14] and vitamin A [15] which all of them found to improves humoral immune response and presences of them together such as ginger powder and vitamin E exert synergistic action and has positive effect on broiler performance [35].

Colistine sulphate is polymixin antibiotic is widely used in poultry industries specially under Egyptian field, many researchers studied its effect on immune response as Bouskra, et al. [36] reported that colistin reduced the proliferation of individual lymphoid follicles as colistine targeting the peptidoglycans of Gram-negative bacteria which is essential for development of intestinal lymphoid follicle, author concluded that effect of colistine on Gram-negative bacteria modulating the percentage of immune cells, mainly B-lymphocytes. On the other hand use of such polymyxins antibiotic found to improves humoral immune response against viral respiratory diseases in poultry [10]. Moreover the use of such antibiotic as vaccine adjuvant found it stimulating of more humoral immune response [11, 31 and 32], our results are parallel was those stated that colistin sulphate improves humoral immune response.

Regarding histopathological findings revealed that control negative group (1) did not show any pathological changes during whole of the experiment, while bursa of control positive vaccinated group showed moderate hyperplastic activity of the lymphoid follicle (fig 3) while spleen showing severe hyperplastic activity of the lymphoid follicle (fig 4), this results was matched with Amer [37] who reported the histopathological changes induced by some AI vaccines in laying hens, and broilers [31]. Group (5) received colistin alone showing vacuolar degeneration in the hepatocytes by the end of the experiment (fig 5) this results indicate that colistine has adverse effect on hepatocytes this results was parallel with Ibrahim et al. [9] who reported hepatotoxicity and nephrotoxicity of colistin antibiotics, in spite of that oral administration of colistin result in little residues in liver, kidney, lung and thigh muscle [38] prolonged use of such antibiotic under our experimental condition and field conditions may resulting in adverse conditions. bursa of Fabricius of group (7) received prebiotic mixture showing severe hyperplastic activity of the lymphoid follicle and the epithelial lining showing hyperplastic activity forming finger like projection (fig 6) this results indicate immune stimulant activities induced by prebiotics mixture this was parallel with [39] who stated that garlic oil boosts immune system, other researchers reported that garlic oil improved broiler chicken immune response through increased neutrophil percentage and weight of immune organs when compared with the control group [40]. Moreover; Ghasemi and Taherpour [41] reported that ginger essential oil improves bursal weight and secondary antibody response against viral vaccines. Control positive group vaccinated non treated liver showing severe congestion of the central vein and sinusoids, vacuolar degeneration of the cytoplasm in the hepatocyte with disorganization of the hepatic cord (fig 7), this results was matched with results found by Zeinab et al. [42] who reported that antimicrobial agents in inactivated AI-ND or IBD vaccines not affect vaccine potency but maybe responsible for degeneration of hepatic cells and congestion of the central vein with inflammatory cell infiltration and congestion of blood vessels.

From the present study it could be concluded that the use of antibiotics in poultry industries is of great value but it could affect humeral immune response against poultry vaccines, moreover prebiotics is of great value in poultry industries as it improves immunity and dilute hazard effects of antibiotics on vital organs. Under the condition of our work it was noticed that the usage of single inactivated vaccines against

two viruses resulting in better immune response than combined inactivated vaccine this results needs further investigation.

**Table (1): Treatment of chicken groups vaccinated with AI-ND inactivated vaccine.**

Gr No	Vaccination	Treatment
1	-	Negative control
2	+	tilmicosin - colistin
3	+	tilmicosin
4	+	tilmicosin+ prebiotic
5	+	colistin
6	+	Colistin - prebiotic
7	+	prebiotic
8	+	Vaccinated with combined ND-AI vaccine non treated
9	+	Vaccinated with single inactivated AI and ND vaccine with no treatment

**Table (2): Main HI titers against ND in vaccinated, medicated and control broiler chicken groups (n= 15).**

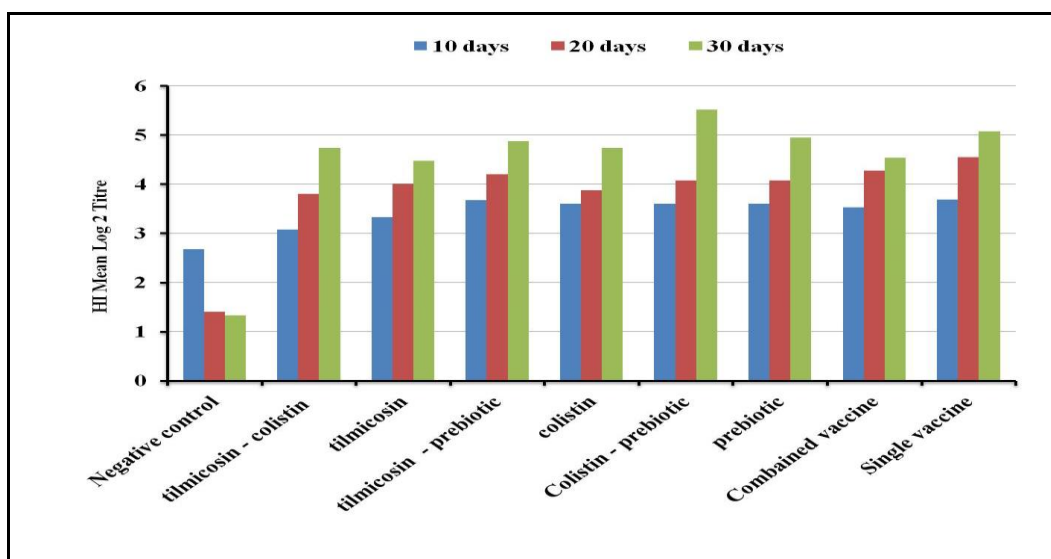
Gr No	Treatment	Age /Days	Disruption of HI log <sub>2</sub> titre reference number							Mean ± SD
			0	2	3	4	5	6	7	
1	Negative control	0		4	6	4	1			3.13 1.95
		10	2	4	5	3	1			2.67 1.40
		20	5	5	4	1				1.73 1.39
		30	6	7	2					1.33 1.18
2	tilmicosin - colistin	10	1	4	5	2	3			3.07 1.38
		20		2	4	5	3	1		3.80 1.15
		30		1	1	4	5	3	1	4.73 1.28
3	tilmicosin	10		3	6	4	2			3.33 0.89
		20		1	4	5	4	1		4.00 1.07
		30		1	1	3	5	4	1	4.47 1.41
4	tilmicosin - prebiotic	10		2	4	7	2			3.67 1.05
		20		1	3	4	6	1		4.20 1.08
		30			2	3	6	2	2	4.87 1.13
5	colistin	10	1	4	5	3	2			3.60 1.58
		20	1	1	3	4	4	2		3.87 1.31
		30		1	1	6	5	2	1	4.74 1.03
6	Colistin - prebiotic	10		2	4	5	2	2		3.60 1.58
		20	1	3	2	4	4	1		4.07 1.25
		30			2	1	5	3	4	5.51 1.62
7	prebiotic	10		2	5	5	3			3.60 1.59
		20		1	3	7	2	2		4.07 0.99
		30		1	1	6	2	3	2	4.95 1.46
8	combined inactivated vaccine	10		3	4	6	1	1		3.53 1.13
		20		2	2	4	4	3		4.27 1.33
		30		1	3	3	5	1	2	4.53 1.38
9	single inactivated vaccine	10		2	5	6	1	1		3.68 1.22
		20		1	2	4	5	3		4.55 1.42
		30		1	2	1	5	3	3	5.07 1.53



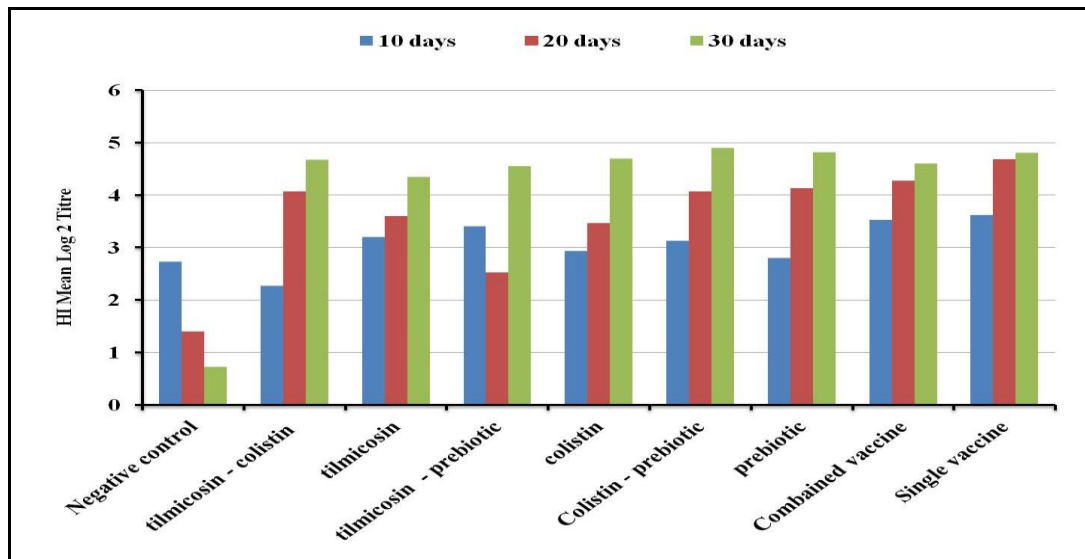
Table (3): Main HI titers against *Avian Influenza* H5N1 in vaccinated , medicated and control broiler chicken groups (n= 15).

Gr No	Treatment	Age /Days	Disruption of HI log <sub>2</sub> titre reference number						Mean ± SD
			0	2	3	4	5	6	
1	Negative control	0		2	3	8	2		3.67 0.89
		10	2	1	9	3			2.73 1.22
		20	7	4	3	1			1.40 1.45
		30	10	4	1				0.73 1.10
2	tilmicosin - colistin	10	4	3	5	2	1		2.27 1.62
		20		2	4	8		1	4.07 0.69
		30			2	3	8	2	4.67 0.90
3	tilmicosin	10	2	1	5	4	3		3.20 1.57
		20	1	2	3	8	1		3.60 0.74
		30		1	5	6	2	1	4.35 0.82
4	tilmicosin - prebiotic	10		2	5	5	3		3.40 0.74
		20	2	5	4	1			2.53 1.73
		30		1	4	5	4	1	4.55 1.10
5	colistin	10	2	3	6	3	1		2.93 1.45
		20		1	2	8	2	2	4.13 1.06
		30		2		4	7	2	4.69 0.89
6	Colistin - prebiotic	10	2	1	5	5	2		3.13 1.50
		20	1	2	4	4	4		3.47 1.41
		30		1	2	2	7	3	4.88 1.19
7	prebiotic	10	2	1	8	4			2.80 1.24
		20		1	4	5	2	3	4.13 1.25
		30			3	4	6	2	4.82 0.91
8	combined inactivated vaccine	10	2		4	5	4		3.53 1.64
		20		2	2	3	6	2	4.27 1.28
		30		1	2	3	5	4	4.60 1.24
9	single inactivated vaccine	10		2	4	4	5		3.62 0.92
		20		1	1	4	7	2	4.68 1.31
		30			2	4	6	3	4.81 1.44

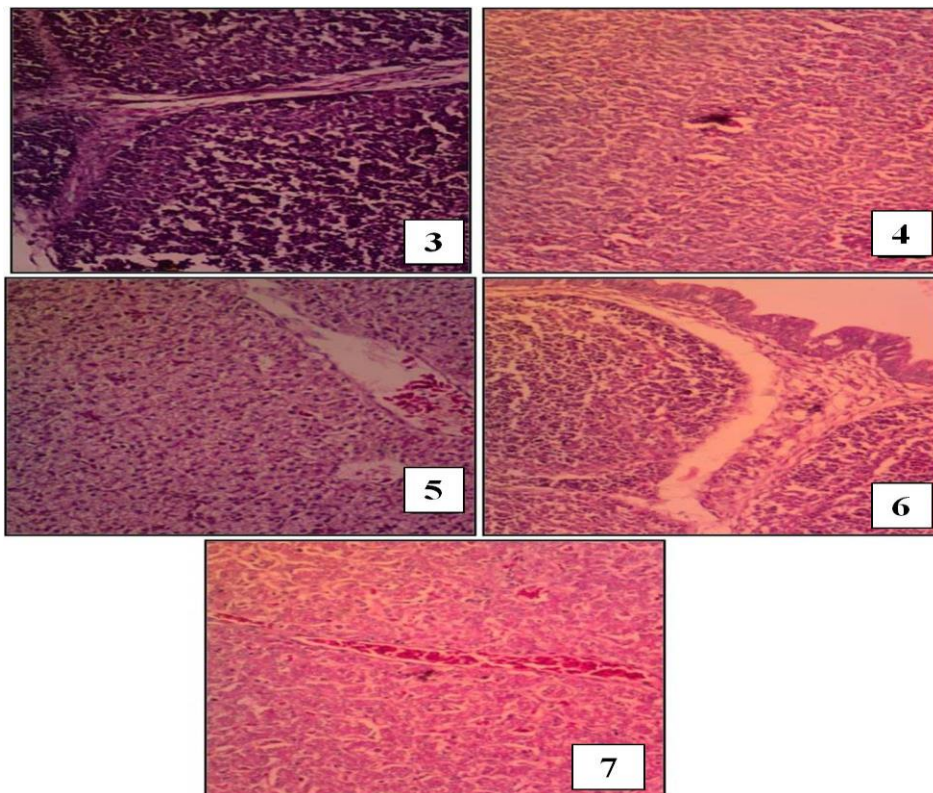
Fig (1): Levels of HI titers against ND in vaccinated , medicated and control broiler chicken groups



**Fig ( 2 ):** Levels of HI titers against AI in vaccinated , medicated and control broiler chicken groups



**Fig (3-7):** Bursa, spleen and liver stained sections of vaccinated , medicated and control broiler chicken groups. (H&E X 200).



**Fig (3):** bursa showing moderate hyperplastic activity of the lymphoid follicle.

**Fig (4):** Spleen showing severe hyperplastic activity of the lymphoid follicle.

**Fig (5) :** liver group 7 showing vacuolar degeneration in the hepatocytes cytoplasm.

**Fig (6):** Bursa: showing severe hyperplastic activity of the lymphoid follicle and the epithelial lining suffering from hyper plastic activity forming finger like projection.

**Fig (7):** Liver of Control positive (vaccinated non treated): showing severe congestion of the central vein and sinusoids, vacuolar degeneration of the cytoplasm in the hepatocyte with disorganization of the hepatic cord.



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