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Efficacy of Five Commercial Available Inactivated Avian Influenza Vaccines in both Specific Pathogen Free (SPF) and Commercial Broiler Chicks against Challenging with the Current Recently Isolated HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) Field Strain

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ABSTRACT

The endemic highly pathogenic H5N1 avian influenza viruses (A/H5N1) of clade 2.2.1 in Egypt compromise the poultry industry and pose a serious public health threat. In spite of vaccination, infections of commercial poultry flocks have been frequently reported. The 2.2.1.2 viruses were isolated from vaccinated commercial poultry and are postulated to be immune escape variants (IEV). In a trial to control the wide spread of Highly pathogenic avian influenza (HPAI) H5N1 virus outbreaks among poultry flocks in Egypt this study has been pursued to Evaluate the efficacy of possible available AI (H5N1, H5N2 and H5N3) vaccines against challenging with the current recently isolated HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) field strain this trail was planned to explain why some poultry farms are vaccinated against AI and suffered from outbreaks of HPAI infection. in this study 5 commercially available inactivated avian influenza vaccines were evaluated for their efficacy using both SPF and broiler chickens, SPF groups were vaccinated at 21st day old while broiler groups were vaccinated at 10th day old to simulate field condition, the groups were serologically monitored on a weekly basis post vaccination (PV) using HI test, then challenge test were conducted at 28th PV for SPF groups and at 28th day old for broiler groups using the recently isolated (A/duck/Egypt/CLEVB-24_N00238/2015) field strain s challenge virus, oropharyngeal swab samples were collected for detection of virus shedding titer by real time RT- PCR). The results of HI against the homologous antigens for each vaccine showed positive seroconversion for the 5 H5 AI vaccines in both SPF and broiler chickens, Meanwhile only 3 H5 vaccines included positive seroconversion against the heterologous A/duck/Egypt/CLEVB-24_N00238/2015 HPAI antigen with mean HI titers of 5.9, 4.7 and 3.9 log2 in broiler chickens and 7.5, 5.1 and 4 log2 in SPF chickens for poulvac H5N3, Nobilis H5N2 and Re-5 H5N1 AI vaccines respectively. While the two local Re-H5N1 AI vaccines included HI titers < 3 (negative seroconversion) in both broiler and SPF chickens. These mean HI values were reflected on the protection percentages which were 90%, 85%, 85%, 50% and 40% in broiler chickens and 90%, 85%, 90%, 55% and 45%, in SPF chickens vaccinated with poulvac H5N3, Nobilis H5N2, Re-H5N1, Mevac H5N1 and serovac H5N1 AI vaccines, respectively. Finally concerning the reduction on the challenged HPAI H5N1 virus shedding, only the same 3 vaccines (poulvac H5N3, Nobilis H5N2 and Re H5N1 – induced significant reduction in the titer of the shedding virus (more than 2 logs) compared to the non-vaccinated challenged groups in both broiler and SPF chickens while the rest two did not provide significant reduction in the titer of shedding virus compared to the non-challenged control group in both boiler and SPF chickens.

Key words: Highly pathogenic avian influenza H5N1, Antigenic drift, Vaccination, Egypt, Broiler breeders.

Introduction

In Egypt the classic group of clade 2.2.1 that was introduced in 2006 remained stable through 2009 and represented the original viruses known at that time. The variant clade 2.2.1.1, which emerged in late 2007 from vaccinated commercial poultry, was subdivided into 2 clusters from 2008 to 2011 (2.2.1.1 and 2.2.1.1a). The first cluster emerged in late 2007 (2.2.1.1) and remained until 2009, while the second cluster (2.2.1.1a) emerged in 2008 and remained until 2011. Since then, these variant clusters have not been detected. In 2008, the classic viruses evolved into a new clade 2.2.1.2 due to gradual accumulation of genetic mutations in the HA protein, and was the dominant cluster between 2009 and 2014 in both the household and commercial poultry sectors irrespective of their vaccination status Arafa *et al.,* (2016),. Since late 2014, the incidence of H5N1 outbreaks among poultry flocks has increased as FAO stated that during December 2014 and May 2015, 492 poultry outbreaks were notified in Egypt, which a large

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increase is compared to the 44 poultry outbreaks reported during the same period the year before. This outbreaks were reported among vaccinated poultry in commercial farms and households as well as in unvaccinated backyard poultry (FAO reports, 2014).

In spite of vaccination of poultry flocks with H5 Vaccines there is a Nemours HPAI H5N1 outbreaks were reported from December 2014 till May 2015, more than 19 H5 vaccines were commercially available in Egypt represented about 12 seed virus strain (unpublished data). The aim of our study id to evaluate the efficacy of possible available AI (H5N1, H5N2 and H5N3) vaccines against challenging with the current recently isolated HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) field strain this trail was planned to explain why some poultry farms are vaccinated against AI and suffered from outbreaks of HPAI infection.

Materials and Methods

Experimental chicks and housing

Two hundred ten SPF one day old (DO) chicks were kindly supplied from Qum Oshim SPF farm at el Fayoum governorate. The chicks were housed in poultry BSL3 chicken isolators all over the experimentation period. The chicks were reared under proper Hygienic conditions ventilated under negative pressure with HEPA- filtered air and maintained under continuous lightening, feed and water will be supplied ad libitum, the chicks were placed properly and monitored daily for mortality and health status and Two hundred and fifteen commercial broiler day old (DO) chicks of Ross breed , were kindly supplied from El Wadi Company, carrying significant level of maternally derived antibodies (MDA) to H5N1 AIV, representative of the common situation in Egypt. The chicks were housed in poultry cages and were, placed properly and monitored daily and the chicks were vaccinated against broiler viral diseases.

Vaccines and Viruses:

A- Vaccines and antigens:

1- Inactivated reassorted H5N1 Avian influenza virus vaccine prepared from (H5N1 subtype, Re-5 (A/duck/Anhui/1/2006 clade 2.3.4) strain, produced by Merial Nanjing Animal Health Co.
2- Poulvac FluFend H5N3 RG prepared from rg-A/ck/VN/C58/04 strain with N3 gene from A/Duck/Germany/1215/73 (H2N3) and six internal genes from PR8 vaccine strain avian influenza virus of H5N3 subtype (strain rg-A/ck/VN/C58/04), produced by Fort Dodge Animal Health.
3- SER-VACCFLU prepared from RGA/ chicken/ Egypt/ M2583D/2010 (H5N1) Clade 2.2 strain and produced by Veterinary Serum and Vaccine Institute, Cairo, Egypt.
4- Nobilis Influenza H5N2 prepared from H5N2, LP (A/duck/Potsdam/1402/86) strain, produced by Intervet, Boxmeer
5- MEFLUVAC prepared from 2 reverse genetic strains RGA /chicken /Egypt/ Q1995D /2010 (H5N1), RGA/ chicken/ Egypt/ M2583D/2010 (H5N1), produced by ME-VAC, Company, Cairo, Egypt.

Other vaccines used in this study:

Variant IB vaccine, IBD vaccine and ND vaccine

B- Viruses:

The Egyptian recently isolated and fully characterized HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) field strain with accession no EPI579780 on GISAID which was isolated from duck flock located in Monufia and has been fully identified, used as challenge virus and also used as HI antigen after treatment with Binary ethylenimine (BEI). As well as, the vaccinal homologous antigens were used.

Experimental design:

Two experiment was carried out in this study the first one carried out on the SPF chickens as At 21th day old the chickens were classified and identified into 7 groups (from gp1 to gp7) each of 30 chickens, from group 1 – group 5 the chickens were injected with 0.5 ml S/C with the tested 5 inactivated reassorted

H5N1 Avian influenza, Poulvac FluFend H5N3, SER-VACCFLU, Nobilis Influenza H5N2 and MEFLUVAC vaccines respectively ,while group 6 considered as challenged non-vaccinated control group (positive control group) and group 7 considered as non-vaccinated non challenged (negative control group) , Vaccination was conducted for all inactivated vaccines based on manufacturer recommendation dose. Daily observation of all experimental groups from the beginning of the experiment for reporting of any clinical signs, recording of any mortalities.

Challenge test was conducted at 28 day post vaccination on 20 chicken from each group as well as group 6 using the HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) clade 2.2.1.2 virus, Each challenged chicken was inoculated intranasally with 100ul contain 10^6 EID50/chicken, Challenge experiment was conducted inside BSL3 chicken isolators , All chickens were subjected to daily observation and monitoring for 10 days post challenge (DPC) in order to report the clinical sings as well as record mortalities and detection of virus shedding titer.

The second one was conducted on commercial broiler chicks - to simulate the field condition- At 10^{th} day old the chicks were classified and identified into 7 groups (from gp1 to gp7) each of 30 chicks and they were treated as the first one while the challenge test was conducted at 28^{th} day old

Serology:

Individual serum samples corresponding to each ten blood samples were collected from each group (1-7gp) at 7^{th}, 14^{th} ,21^{th} and 28^{th} days PV for post vaccination monitoring in the first experiment, while it was collected at 1^{st}, 7^{th}, $14^{th,}$ ^{21th} and 28^{th} day old and from non-vaccinated non challenged group (gp7) for waning up of the maternally derived antibodies. Serum samples were subjected for haemagglutination inhibition test (HI) according to OIE diagnostic manual 2015 using the homologous and heterologous HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) prepared antigen by using standard 4 HAU of the antigen. Data of HI results were statistically analyzed.

Detection of Virus shedding titer:

Individual oropharyngeal swab samples (10 individual from each group) were collected from all vaccinated challenged as well as non-vaccinated challenged groups in both experiment at 3^{rd}, 5^{th}, 7^{th} and 10^{th} day post challenge (DPC). Swab samples were prepared to be suitable for testing using real time Rt-PCR.

RNA was extracted from the oropharyngeal swabs using QIAamp Viral RNA Mini Kit that supplied from (Qiagen, Valencia, Calif., USA) Cat. No. 52906. Samples were amplified using Invitrogen superscript® III platinum® one- step Quantitative RT-PCR Cat. No 11732-088 to investigate the presence or absence of AIV-H5N1 following the manufacture instructions using primers and probe and reaction condition as described by Spackman *et al*., (2002).

RRT-PCR for detection of the Shedding titer (PCR copies/ml) was conducted targeting theH5 gene and results of Cq values were calculated against challenge virus standard curve. Demonstration of reduction in replication and shedding titers of virus from respiratory tract should be at a minimum of (102) 2 logs (100 fold) less virus in vaccinated compared to non-vaccinated chickens Suarez *et al*., (2006), Mean shedding titer= sum of shedding titer/number of shedders birds.(10from each group).

Statistical analysis:

Statistical analysis of log2-transformed HI titers in serologic tests was done with ANOVA and T test at a 95% level of significance, and least significant differences were used to determine statistically significant differences between means.

Results and Discussion

The effectiveness of vaccination was evaluated on the basis of clinical protection (morbidity and mortality) and measurement of virus shedding after challenge. Immune response to vaccination was by evaluation the serological response (mean HI titer).

In the current study there is no observation of any clinical sings or mortalities till time of challenge test. The serological response (mean HI titer) of the vaccinated SPF chickens against the homologous antigen showed positive seroconvertion for 5 tested vaccine as it were 8.5, 9.8, 4.5,9.1 and 5.2 at 28^{th} PV for gp1,gp2,gp3,gp4 and gp5 respectively.

The recorded serological response (mean HI titer) of the vaccinated SPF chickens groups against the newly isolated heterologous HPAI A/duck/Egypt/CLEVB-24_N00238/2015 antigen are illustrated in Table (1) and Fig (1) , The mean log 2 HI antibody titer at 4th week PV induced by poulvac H5N3 , Nobilis Influenza H5N2 and Reassortant H5N1 were significantly higher (P<0.05) than those induced by the two local H5N1 vaccines, the latter two vaccines failed to induce positive seroconvetion with the heterologous the HPAI H5N1 (A/duck/Egypt/CLEVB-24_N00238/2015) antigen.

Table 1: Mean HI antibody titers (Log 2) of SPF chickens vaccinated with different AI vaccines tested using the prepared hetrologous A/duck/Egypt/CLEVB-24_N00238/2015 HPAI antigen:

Mean HI results				
DPV / Group	7th DPV	14th DPV	21th DPV	28th DPV
Group1	0.6 ±0.69	1.5 ±0.97	2.8 ±1.03	4 ±0.81*
Group 2	0.8 ±0.79	1.6 ±0.84	4.9 ±0.73	7.5 ±0.84*
Group 3	0	0	0.3 ±0. 48	0.6 ±0.69
Group 4	0.7 ±0.67	1.5 ±0.52	3.1 ±0.73	5.1 ±0.73*
Group 5	0.3 ±0.48	0.7 ±0.48	1.2 ±0.78	2 ±0.81
Control	0	0	0	0

The arithmetic mean and ±standard deviation of HI titers are shown
**= statistically significant difference at P<0.05.*

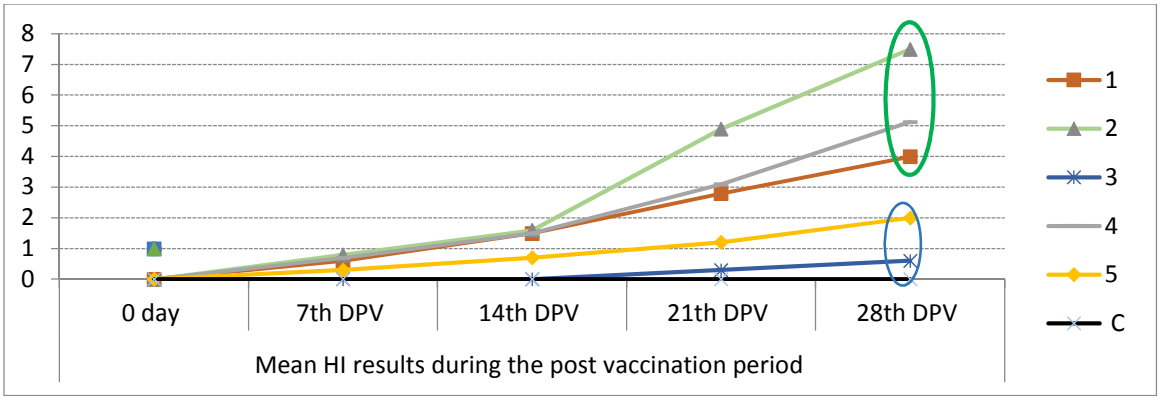

Fig. 1: Mean HI antibody titers (Log 2) against the hetrologous A/duck/Egypt/CLEVB-24_N00238/2015 of SPF chickens vaccinated with different AI vaccines. The results of mean HI antibody titer against the heterologous A/duck/Egypt/CLEVB-24_N00238/2015 indicates that group 2 which vaccinated with PoulvacFlufend(B) has the highest mean of the antibody titer followed by group 4 which vaccinated with Nobilis Influenza H5N2(D) then group 1 which vaccinated with Reassortant H5N(A) then. The lowest groups were group 3 and 5 which vaccinated with inactivated Servac Flu and ME FLUVAC respectively.

The arithmetic mean log2 HI titers were 7.5, 5.13 and 4 for poulvac H5N3, Nobilis Influenza H5N2 and Reassortant H5N1 AI vaccines, respectively compared with 2 and 0.6 log2 for local H5N1 vaccines.

After challenge which was conducted at 28th PV the protection percentage were very similar to the HI pattern (Table 2) as the protection percentage was 90% , 90% ,85% , 55 % and 45% for inactivated reassorted H5N1 Avian influenza, Poulvac FluFend H5N3RG, Nobilis Influenza H5N2 , MEFLUVAC and SER-VACCFLU respectively. And this is in agreement with (Tian *et al*., 2005) as he mentioned that 4 log 2 or 5 log 2 (Bertelsen *et al*., 2007) titers are considered protective titers, Furthermore, HI antibody titer of 1/16 is considered a minimum titer that could protect chickens from HPAI virus infection related death and it is correlated with the antigen levels of vaccines (Sasaki *et al*., 2009).

And this mean that the inactivated reassorted H5N1 Avian influenza, Poulvac Flu Fend H5N3RG , Nobilis Influenza H5N2 vaccines are immunogenic and protective.

On the same context the reduction of the challenged virus shedding titer was significantly higher for the same 3 protective vaccines when compared to challenged non vaccinated positive control group as the difference in the mean of virus shedding titer was 2.7, 2.4 and Table (3) for Poulvac FluFend H5N3RG, Nobilis Influenza H5N2 and reassorted H5N1 respectively and it was higher than the other 2 vaccines, the later 2 vaccines did not induce any significant reduction of the challenged virus shedding titer compared to challenged non vaccinated positive control. As Suarez *et al*., (2006) concluded that the demonstration of

reduction in replication and shedding titers of virus from respiratory tract should be at a minimum of 2 logs10 (100 fold) less virus in vaccinated compared to non-vaccinated chickens.

Table 2: Summary of data record of SPF chickens after challenge with A/duck/Egypt/CLEVB-24_N00238/2015 HPAI virus and mean HI titer at 28thDPV:

Group	No. of challenged birds	Total mortalities	Protection (%)	Mean HI titer at 28th dpv	*Difference in mean of virus shedding titer
Group1	20	2	90	4 ±0.81	2.35
Group2	20	2	90	7.5 ±0.84	2.7
Group3	20	11	45	0.6 ±0.69	1
Group4	20	3	85	5.1 ±0.73	2.4
Group5	20	9	55	2 ±0.81	1.2
Group6	20	20	0.0	0	0
Group7	0	0	-	0	-

**Difference in mean of virus shedding titer between vaccinated groups and challenged non vaccinated control group which should be at a minimum of (102) 2 logs (100 fold) less virus in vaccinated compared to non-vaccinated chickens Suarez et al., (2006)*

Table 3: Results of virus shedding of SPF chickens after challenge with HPAI A/duck/Egypt/CLEVB-24_N00238/2015 virus: at 3rd, 5th, 7th, 10thdpc:

	Mean Shedding of challenge virus titer (Log10 copies)				
DPC / Group	3rddpc	5thdpc	7thdpc	10th dpc	**cumulative mean
Group 1	3.5 ±0.48*	3.3 ±0.24	3.1 ±0.31	2.5 ±0.36	3.1
Group 2	3.4 ±0.41	3 ±0.31	2.5 ±0.34	2.1 ±0.68	2.75
Group 3	4.9 ±0.46	4.8 ±0.11	4.2 ±0.54	4 ±0.34	4.775
Group 4	3.6 ±0.51	3.2 ±0.42	2.8 ±0.24	2.3 ±0.39	2.97
Group 5	4.5 ±0.49	4.5 ±0.34	4 ±0.38	3.9 ±0.27	4.22
Group 6(Control+)	5.4 ±0.4	-	-	-	0
Group 7(Control-)	-	-	-	-	-

**Data represent arithmetic mean ±standard deviation of H5 gene copies in ml of swabs (Arithmetic mean shedding titer= sum of shedding titer (log10 HPAI H5N1 virus titer) /number of shedders birds).*
***Cumulative mean= cumulative mean shedding titer of four days.*

The poor efficacy of the local vaccines were not fully investigated in spite of the higher identity to our newly challenged virus than the other 3 vaccines and to explain these results: firstly sequence similarity is not the sole determining factor predicting a vaccine protective potential against the disease or vial shedding (Pfeiffer *et al*., 2012). so the significantly higher HI titer , protection percentage induced by reassorted H5N1 Avian influenza, Poulvac FluFend H5N3RG, Nobilis Influenza H5N2 vaccines could be due to the variation in the antigenic mass of the viruses used in these vaccines which proved to be significantly higher than that of the 2 other vaccines. On the other hand, other two factors including the adjuvant used in the formulation of these vaccines could be differ (type, quality and its effect on cellular immunity), also the antigenicity of the HA of such vaccines our explanation is in agreement with that of (Swayne *et al*., 1999) who found that the remarkable immunogenicity of the h5 vaccine could be attributed to the proprietary adjuvant used in the formulation of such vaccine, the antigenic mass or the inherent antigenicity of the HA protein itself.

Nearly the same results were obtained in the experiment of commercial broiler Tables (4, 5 & 6) and fig (2).

Table 4: Mean HI antibody titers (Log 2) of commercial broiler chickens vaccinated with different AI vaccines tested using the prepared heterologous A/duck/Egypt/CLEVB-24_N00238/2015 HPAI antigen:

	Mean HI results				
D.O Group	1st D.O	7th D.O	14th D.O	21st D.O	28th D.O
1	5.4±0.69	3±0.66	2.1±0.73	3±0.66	3.9±0.73*
2	5.4±0.69	3±0.66	2.8±0.63	4.2±0.78	5.9±0.87*
3	5.4±0.69	3±0.66	2±0.81	1.2±0.42	0.5±0.52
4	5.4±0.69	3±0.66	2.4±0.84	3.3±0.67	4.3±0.67*
5	5.4±0.69	3±0.66	2±0.66	1.8±0.42	2.3±0.67
6 control	5.4±0.69	3±0.66	2±0.47	1.2±0.42	0

The arithmetic mean and ±standard deviation of HI titers are shown
**= statistically significant difference at P<0.05*

Although the challenge was done at the 18th DPV. The MDA found to affect the level of PV HI antibodies at the 1st two weeks PV but not affect the protection percentage by 5 vaccines compared to the results of the same 5 vaccines in the SPF chickens. As the protection percentage in the commercial broiler chickens which vaccinated at 10th DO was 90%, 85%, 85%, 50 % and 40% for Poulvac FluFend H5N3RG, reassorted H5N1 Avian influenza, Nobilis Influenza H5N2, MEFLUVAC and SER-VACCFLU respectively. The cause of absence of effective interference of the MDA may be attributed to the time of vaccination or the antigen used in testing the serological immune response.

Table 5: Summary of data record of broiler chickens after challenge with A/duck/Egypt/CLEVB-24_N00238/2015 HPAI virus:

Group	No. of challenged birds	Total mortalities	Protection (%)	Mean HI titer at 28th dpv	*Difference in mean of virus shedding titer
Group1	20	3	85	3.9 ±0.73	2.37
Group2	20	2	90	5.9 ±0.87	2.75
Group3	20	12	40	0.5 ±0.52	1.1
Group4	20	3	85	4.3 ±0.67	2.8
Group5	20	10	50	2.3 ±0.67	1.35
Group6	20	20	0.0	0	0
Group7	0	0	-	0	-

**Difference in mean of virus shedding titer between vaccinated groups and challenged non vaccinated control group which should be at a minimum of (102) 2 logs (100 fold) less virus in vaccinated compared to non-vaccinated chickens Suarez et al., (2006).*

Table 6: Results of virus shedding of commercial broiler chickens after challenge with HPAI A/duck/Egypt/CLEVB-24_N00238/2015 virus: at 3rd, 5th, 7th, 10th dpc:

	Mean Shedding of challenge virus titer (Log10 copies)				
DPC Group	3rddpc	5thdpc	7thdpc	10th dpc	**cumulative mean
Group 1	3.9±0.22*	3.6±0.31	3.3±0.49	2.7±0.44	3.375
Group 2	3.8±0.27	3.2±0.24	2.7±0.29	2.3±0.41	3
Group 3	5±0.34	4.9±0.37	4.7±0.38	4±0.59	4.65
Group 4	3.7±0.21	3.1±0.29	2.6±0.39	2.4±0.35	2.95
Group 5	4.9±0.42	4.6±0.39	4.1±0.24	4±0.38	4.4
Group 6(control+)	5.7±0.22	-	-	-	
Group 7(control-)	-	-	-	-	

** Data represent arithmetic mean ±standard deviation of H5 gene copies in ml of swabs (Arithmetic mean shedding titer= sum of shedding titer (log10 HPAI H5N1 virus titer) /number of shedders birds).*
***Cumulative mean= ` mean shedding titer of four days.*

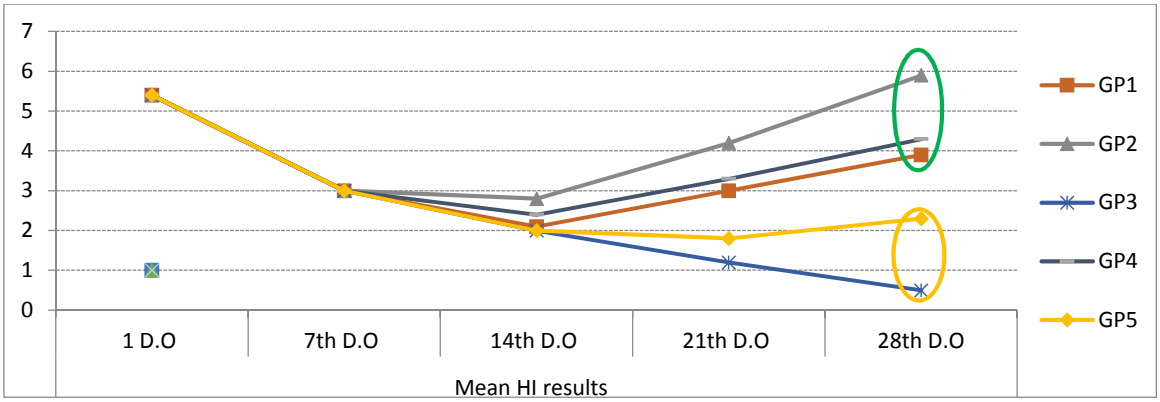

Fig. 2: Mean HI antibody titers (Log 2) of commercial broiler chickens vaccinated with different AI vaccines against the prepared hetrologous A/duck/Egypt/CLEVB-24_N00238/2015 HPAI antigen: -The results of mean HI antibody titer against the heterologous A/duck/Egypt/CLEVB-24_N00238/2015 indicates that group 2 which vaccinated with PoulvacFlufend(B) has the highest mean of the antibody titer followed by group 4 which vaccinated with Nobilis Influenza H5N2(D) then group 1 which vaccinated with Reassortant H5N(A). The lowest groups were group 3 and 5 which vaccinated with inactivated Serovac Flu and ME FLUVAC respectively.

Conclusion

More attention must be directed toward the measurement of the antigenic mass of the HA protein in the inactivated H5 vaccines regarding the evaluation of such vaccines, the improvement of the adjuvant type and quality has to be taken in consideration when talking to the production of H5 vaccines finally vaccination of H5 vaccines in broiler flocks must done not earlier than 10 day of age to avoid interference MDA.

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