Protective efficacy of two H5 inactivated vaccines against challenge with 2008 and 2010 representative HPAI viruses under field condition

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Abstract
Since 2006 Egypt had faced an extensive epidemic of H5N1 highly pathogenic avian influenza (HPAI) with a huge number of outbreaks both in commercial and backyard poultry sectors. AI vaccination strategy has been applied since end of March from same year as supportive tool to control HPAI and from this date, Egypt has embarked on mass vaccination strategy which is essential for the control and possible eradication of HPAI. The present study was performed to evaluate the protective efficacy of most common used inactivated AI H5 vaccines (H5N2/Mexico) and (Re1 H5N1/Chinese) against two different HAPI Egyptian variant viruses isolated in 2008 and 2010, based on clinical protection, immune response and ability to reduce amount of challenge virus shedding.
Ten thaoutnad broiler chickens were divided into 2 groups vaccined at seven days old with H5N2 or re-H5N1 commercial vaccines plus other non vaccinated group. Weekly blood samples were collected for humeral immune response monitoring. At 35 days age, only 30 vaccinated birds from each group and 20 non vaccinated birds were divided into 6 different groups and challenged intranasal with 10^6 EID50/0.1 ml using both 2008 and 2010 HPAI H5N1 variant viruses.
All vaccines used in this study were immunogenic in chickens and provide good HI log2 titers using their homologous vaccinal antigens, but there was no cross reaction with the Egyptian variants H5N1 antigens. Birds vaccinated with H5N2 vaccine had 80-100% mortality, while other re1-H5N1 vaccinated groups had 70–90% mortality when challenged with 2008 and 2010 H5N1 viruses respectively. The highest virus excretion was found in non-vaccinated infected.. Eleven peculiar amino acid substitutions in H5 protein of the variant strain were existed neither in the vaccine strains nor in the earliest H5N1 virus introduced into Egypt in 2006.
In conclusion, these two different vaccine although their high quality and immunogenicity but they were not able to provide good protection under field condition against the current circulated variants H5N1 viruses. Yearly update and reviewing our vaccination strategy is required for better selection of good protective and efficient AI vaccine and vaccination regimes.

Key words "Vaccine, HPAI, Highly Pathogenic Avian Influenza, AI Avian Influenza, Egypt and broiler chicken"

Introduction.

Highly pathogenic avian influenza (HPAI), caused by the influenza A H5N1 virus, poses a significant threat to the poultry industry and humans in Egypt. Since it was first recognized on a larger scale in 2006, the disease become enzootic in poultry throughout Egypt and still circulates in the poultry population. **(Aly *et al.* 2006 a, b).** By med- 2005 a variant strain of HPAI H5N1 virus emerged and killed large numbers of migratory birds in Qinghai, China, and subsequently in Mongolia and southern Siberia and caused infection for wide range of migratory birds and poultry in Europe, Middle East and Africa. **(Chen *et al.* 2006).** Phylogenetic studies have identified to date nine major clades of H5N1 HPAIV of Asian origin **(WHO/FAO/OIE H5N1 Evolution Working Group, 2007).**

Successful control efforts of HPAI H5N1 seems to be difficult due to lack of movement control, random unorganized poultry farm , backyard and live poultry markets, lack of compensation and improper vaccination and culling strategy all these factors shared in the spread and existence of the disease in Egypt **(Abdelwahab *et al*. 2009)** also reported by **(Capua and Alexander., 2006).** HPAI outbreaks become progressively more expensive to be controlled plus their economic impact on poultry producers and consumers is a reflection of the growth of the poultry industry worldwide so that AI vaccines and their field application can be an effective tool within a comprehensive control program **(Swayne, 2004, Swayne and Akey, 2005, Swayne and DKapczynski, 2008).**

Due to the possibility of antigenic drift and shift of AI viruses, random point mutations in the genes coding for the two surface proteins "Hemagglutinin (HA) and Neuraminidase (NA)" are possible, allowing these viruses to escape the existing immunity to current used vaccines. Through this process of antigenic drift and evolving of new variants can cause epidemics in humans and also in poultry such mutations in serotype H5 and H7 of AIV may result in new outbreaks of mild to severe nature. However, the rate of occurrence of antigenic drift among AIVs is usually low in nature, but, the possibilities of rapid mutations is greater in case of genetic reassortment upon infection of multiple serotypes of AIV in chickens **(Sattar *et al*., 2007).**

Numerous small-scale experimental studies using various vaccines against H5N1-HPAI have demonstrated the value of vaccination in protecting poultry from disease and minimizing virus excretion in poultry subsequently exposed to virus **(Swayne, 2003). (Aly *et al*., 2007)** conducted a study to evaluate vaccine immunogenicity by the HI test using the seed strain relevant to each vaccine and the result showed that the vaccine efficacy is dependant on the vaccine antigen and homology to field virus. Efficacy of homologous vaccines has been proven and the level of protection against mucosal infection and subsequent shedding of challenge virus may depend on the degree of sequence similarity between HA gene of vaccine and challenge virus **(Swayne *et al.,* 1999 and Swayne *et al.,* 2000).**

Since the first introduction of HPAI H5N1 in February 2006 in Egypt, many vaccination breakage record in different poultry farms and different poultry sectors (broiler, layer and turkey) which have history of vaccination by different types of commercial available AI vaccines **(Aly et al. 2008, Arafa *et al*. 2009 and Abdelwahab *et al.* 2009).** This indicates the significance of continuous surveillance for evaluating the effectiveness of the available vaccines against the newly variant viruses.

It is important to carry out different field challenge exercise to understand the effectiveness of current used field AI vaccines against the circulated variant HPAI H5N1 viruses in the Egyptian field. This study was conducted to evaluate the protective efficacy of 2 commonly used commercial (re1 H5N1 and H5N2) vaccines against two Egyptian HPAI 2008 and 2010 H5N1 variants to help better evaluation of these vaccines under field conditions.

Materials and Methods.

HPAI H5N1 Challenge Viruses

H5N1 HPAI Egyptian variant strain (A/ Chicken/Egypt/086-NLQP/2008), **(Arafa *et al.* 2009)** and **(**A/chicken /Egypt/1063/2010 H5N), **(Kilany, 2013)** were isolated at National Lab for Veterinary Quality Control on Poultry Production (NLQP), Egypt. The virus titer was $10^{8.6}$ and $10^{7.2}$ EID50/ml with HA titer 256 HAU, respectively.

AI antigens used:

Commercial Vaccines: a) H5N2- Mex commercial vaccine which prepared from (A/ck/ Mexico/232/1994). b) H5N1-Re-1-china commercial vaccine which prepared from (A/ goose/Gd/1/1996). The two vaccines were finally tested for sterility and safety according to standard protocols described by **(OIE, 2009)**.

Experimental Design and Sampling:

At Farm Level: the selected farm located in Al Behera governorate and it is consisted from 2 floors, each floor contain 5,000 broilers (4500 vaccinated and 500 non vaccinated) each house was vaccinated using different vaccine as following 1) Group A: Vaccinated with H5N1-Re-1 China and Group B: Vaccinated with H5N2-Mex.

All the birds were from the some origin, hatch, mixed genders, same age, received the same feed and management and received the same routine vaccination program adopted in the farm without any special interference. All birds were vaccinated against avian influenza following the label or prospect indications of each vaccine at **7 day old with full dose 0.5 ml S/ C**, by the same vaccination team**.** Different blood samples were collected in weekly basis for evaluation the immune response and also tracheal and cloacal swabs were collected direct before transmission to NLQP to be tested for AIV.

Challenge Experiment: The selected birds were divided into 7 groups (Table 1). Ten birds were selected in each group as shown in table (1), all the selected chickens were shifted to chicken isolators in NLQP at 35 day old (4 weeks after vaccination) for challenging with the HPAI H5N1 variant (A/Chicken/Egypt/086 -NLQP/2008) and (A/chicken /Egypt/ 1063/2010 H5N), with dose 0.1 ml of 10^6 EID50/ ml via I/N rout in NLQP.

Clinical manifestations were monitored for 14 day post challenge day post challenge (dpc), also virus shedding checked by testing cloacal and tracheal swabs every 3 dpc from all birds in each group and processed for virus detection by using M gene RRT-PCR standard protocols **(Slomka *et al.*2007)**. Also the dead birds were examined daily for virus detection. Cloacal and tracheal swabs were collected from experimental chicks and placed in 1.5 ml of Phosphate Buffered Saline (PBS), containing antibiotics (Gentamycin 100 mg/ml, Amphoteracin-B 5 mg/ml and Penicillin 150 mg/ml) according to **(OIE, 2009)**.

Hemagglutination Inhibition Test (HI) was conducted to evaluate the seroconversion against different H5 antigens (Ag) (Two Egyptian challenge viruses Ag, H5N1 Chinese Ag and H5N2 Mexican Ag) according to **OIE, (2009).**

Direct Detection of AI-H5N1 Virus Shedding by Using real time RT-PCR (RRT-PCR):

The collected samples were tested by RRT-PCR in individual basis, RNA was extracted from individual cloacal and tracheal swabs from each bird by using either automated virus RNA extraction Kit with M48 instrument (QIAGEN, Valencia, Calif, USA) or MagNA Pure LC Total Nucleic Acid Extraction kit with MagNA Pure LC instrument (Roche, Mannheim, Germany). Samples were amplified using a One-Step RRT-PCR kit (QIAGEN) for influenza A virus according to **(Slomka et al. 2007)**.

Results and Discussion.

Egypt has embarked on a mass vaccination in all poultry sectors. The current policy was to provide and deliver vaccine for household village poultry (Sector 4) free of charge and to permit commercial companies (Sectors 1, 2 and 3) to vaccinate with approved vaccine of

their choice. Vaccination against the disease in Egypt was a supportive tool in order to limit the wide spread of the disease in short period and decrease the impact of the disease on poultry industry and human health **(Aly *et al.* 2008a).** A number of vaccine manufacturers are supplying different inactivated AI vaccines: from different countries, mostly H5N1 and H5N2 vaccines. Approximately 2.5 billion doses from different vaccines were used to control the disease starting from March 2006 until end 2009 **(Aly *et al*. 2008b).**

Genetic and sequence analysis of the Egyptian HPAI viral isolates revealed that there was marked hetergonicity and emergence of a highly diversified variant strains which have low homology percentage with A/Chicken/ Mexico/232/94/CPA H5N2 and A/Goose/ Guangdong/ 1996 H5N1 seed viruses of the currently used vaccines and serious mutations in different antigenic sites which considered these new variants as immune escape mutants **(Abdel-Monem *et al.* 2009; Arafa *et al*. 2009)**. The immune pressure due to vaccination may result in these genetic and antigenic changes in the virus as previously reported in Mexico **(Lee *et al*. 2004; Escorcia et al. 2008; Suarez, 2008).**

This drift can result in a virus being more deficient to escape the host's immunity and ability to control infection, resulting in vaccination failure "vaccines being less protective over time" **(Swayne, 2009)** or total failure of the current vaccine to prevent viral shedding and mortality **(Lee *et al.* 2004).** Therefore, different field challenge studies to evaluate the efficacy of currently used vaccines in Egypt to protect against the newly emergent immune escape mutants in the field are urgently needed.

The Continuous emerging of the endemic HPAI H5N1 virus within vaccinated birds in Egypt in the last five years and emerging of different antigenic and genetic mutant viruses as in group B (B1, B2.1 and B2.2) as we recorded in current study is an opportunity to study the influence of immune pressure on antigenic drift as well as current vaccine efficacy against the newly emerging viruses in different host species. So far, two different viruses are circulating in Egypt; subgroup A2 "classic 2.2.1" strains infect mostly backyard birds and human and the newly emerging subgroup B2.2 "2.2.1 variants" circulate mainly in vaccinated commercial farms **(Abdelwhab *et al.*, 2010 a; Kilany 2013).**

The protective efficacy of two commercial vaccines (H5N2-Mex and H5N1-Re-1-China) were conducted under field condition and challenging the vaccinated birds with two different variant HPAI H5N1 (A/Chicken/Egypt/086-NLQP/2008) and (A/Chicken/Egypt/1063-NLQP/2010).

Protection can be evaluated by measuring prevention of clinical signs and death, quantitative reduction in shedding of challenge virus from respiratory and gastrointestinal tracts and prevention of contact transmission, afford protection indicted by HA-specific humoral immune responses. Reductions in challenge virus replication and titration were assessed by quantitative RRT-PCR. Laboratory assessment of contact transmission is achieved by recording the mortalities and shedding of virus in the contact birds.

Previous studies using inactivated whole H5N1 influenza virus vaccines have indicated that strain-specific neutralizing antibodies provide long-lasting protection against homologous influenza virus challenge but protection is limited against antigenically variant strains (**Subbarao, *et al.*,. 2003).**

These results suggest that currently used vaccines could not provide protection against the currently circulated variant strain in the field and not considered to be a useful tool for the control of H5N1 HPAI epidemic in poultry farms in Egypt and suggest the need to use local homologous vaccine against this group of viruses.

Insufficient protection afforded by the current used vaccines against challenge

with such variant strains in Egypt was experimentally confirmed in chickens (**Kim *et al.*, 2010**). So the protective efficacy of the current available and commonly used H5 inactivated vaccines (Mexican H5N2 and Chinese Re1 H5N1 vaccines) under field condition which have sufficient biosecurity and mangemental level, there were no clinical signs indicating AIV infection or other life threatening infectious agents at the farm. 10000 broiler chickens were housed in two different houses each house contain 4500 bird that vaccinated with either Re-1 H5N1 Chinese or H5N2 Mexican inactivated vaccine at 7 days old plus other 500 sentinel bird that marked with blue paint on its back. In this study, 7 groups, 15 chickens each, were vaccinated at seven days old with one of H5N2 Mexican or H5N1 re 1 Chinese commercial vaccine. At 35 days age, all vaccinated and non vaccinated birds were challenged intranasal with 10^6 EID50/0.1 ml of **A/ chicken /Egypt/086Q/2008 H5N1** (group 1,3 and 5), and **A/chicken/Egypt/ 1063/2010 H5N1 (group 2, 4 and 6) as shown in (table 2)**.

The two AI vaccines (re1 H5N1 and H5N2) were immunogenic in chicken and provide marked humeral immune response when tested by their homologous antigens at 35 days age 5.2 log2 and 5.7log2 respectively, however there was no cross reaction between the commercial vaccines and the both Egyptian challenge viruses antigen as obtained by the hemagglutination inhibition test as shown in **(Figure 1)** these results agreed with **(Kilany *et al*., 2010** and **Abdelwhab *et al*., 2011a)**.

The results of cross HI testing using the local Egyptian challenge viruses antigens indicate that there were no cross antigenic relatedness between the two Egyptian antigens and other vaccinal antigens while there were little cross reactivity between both vaccinal antigens H5N1 and H5N2**. (Abdelwhab 2011; Grund *et al.,* 2011; Kilany *et al.*, 2011; Shany *et al.*, 2011)** estimate that antibody titer post-vaccination using homologous H5N1 and heterologous H5N2 antigens in HI test respectively revealed up to seven-fold differences between the two types of antigens.

During the current study the birds vaccinated with either re1 H5N1 vaccine or H5N2 vaccine and challenged by (**A/ chicken /Egypt/086Q/2008 H5N1)** have had 70-80% mortality respectively while when challenged with (**A/chicken / Egypt/1063/2010 H5N1)** the mortality were 90-100% respectively as shown in **(Table 2 and Figure 2),** the highest virus excretion was found in non-vaccinated infected while both vaccines were capable to minimize amount of virus shedding when compared with vaccinated challenged group by 2-3 log10 EID 50 logs although none of the tested vaccines and vaccination regimes was able to prevent the shedding of challenge virus totally and afford good accepted protection level as shown **(Table 4 and figure 3 and 4)**. **(Grund, et al. 2010,. Kilany, *et al.* 2010,. Abdelwhab, *et al.* 2011a, and Hassan *et al.,* 2012)**.

Based on the clinical signs and mortality data after challenge with two different challenge HPAI viruses there was no vaccine able to afford the required protection after single vaccination at seven days old, so for better protection of chicken against variant HPAI H5N1 challenge multi-dose vaccination regiem at older age is recommended. In order to control the disease, continuous evaluation of the current vaccines in H5N1 endemic countries in the face of virus evolution is a

paramount challenge to mitigate the public health and socio-economic impact of the virus.

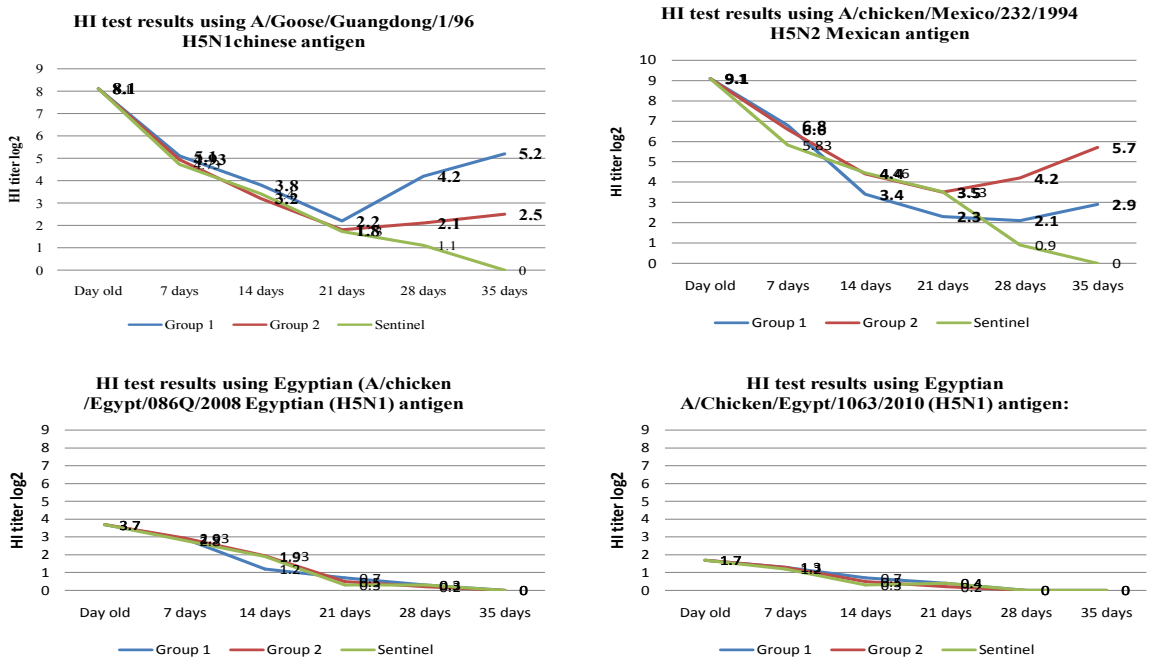
Acknowledgement.

We acknowledge NLQP team and also all the workers in the farm where all trials were conducted .

Table (1). Farm rearing experimental designe.

Group	Type of vaccine used per group	No. of bird in each group
Group (1)	H5N1-Re-1 China	4500
Group (2)	H5N2-Mex	4500
Group (3)	Control negative	500/ house

Results of serology monitoring



bird in group 1 and 2 were vaccinated S/c at 7 days old with dose 0.5ml/ bird.
The HI test done using the 4HAU of tested Ag according to OIE Diagnostic manual 2009.
[1] = Commercial vaccine prepared from Mexican strain (A/ck/Mexico/232/1994 (H5N2))
[2] = Commercial vaccine prepared from Chinese strain (A/goose/Gd/1/1996 (H5N1))

Figure (1). Immune Response of chicks post vaccination.

Table (2). Results of challenge testing in different vaccinated groups.

Group	Type of vaccine	Vaccinated challenged		
		Protection %*	Morbidity	Mortality (MDT)**
Group 1	H5N1-Re-1 China	30%	10/10	7/10 (4.3)
Group 2	H5N2-Mex	20%	10/10	8/10 (3.5)
Group 3	H5N1-Re-1 China	10%	10/10	9/10 (5.5)
Group 4	H5N2-Mex	0%	10/10	10/10 (5.3)
Group 5	Non vaccinated	0%	10/10	10/10 (2.3)
Group 6	Non vaccinated	0%	10/10	10/10 (2.5)
Group 7	Non vaccinated	100%	0/10	0/10

* Protection % = (Number of birds showing clinical signs or died/ total) x 100
** MDT = Mean Death Time.
Group 1, 3 and 5 challenged with HPAI H5N1 Egy 2008.
Group 2, 4 and 6 challenged with HAPI H5N1 Egy 2010.
Group 7 Non challenged.

Table (3). Summary of Mortality% and protection %.

Group	1dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc	9 dpc	10 dpc	Protection %
Group1	*0/10	0/10	0/10	1/10	3/10	5/10	5/10	6/10	7/10	7/10	30%
Group2	0/10	1/10	3/10	5/10	6/10	7/10	7/10	8/10	8/10	8/10	20%
Group3	0/10	0/10	1/10	2/10	3/10	5/10	6/10	8/10	9/10	9/10	10%
Group4	0/10	1/10	2/10	3/10	5/10	7/10	9/10	10/10	10/10	10/10	0%
Group5	0/10	2/10	7/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	0%
Group6	0/10	3/10	8/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	0%
Group7	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	100%

*Number of dead birds/group

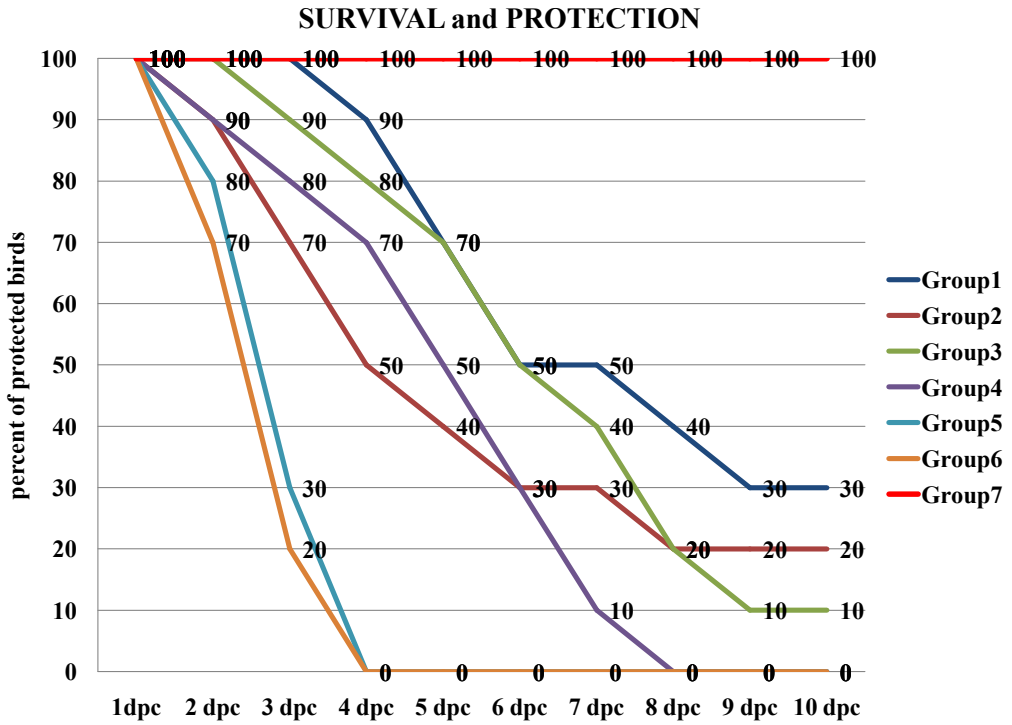


Figure (2). Cumulative mortality and protection%.

Table (4). Cumulative results of virus shedding after challenge.

Group Number	Shedding of challenge viruses									
	2 dpc		**3 dpc		4 dpc		6 dpc		10 dpc	
	Log10 copies	Percent	Log10 copies	Percent	Log10 copies	Percent	Log10 copies	Percent	Log10 copies	Percent
Group1	*2.75	80%	NA	NA	3.7	90%	3.8	100%	0	0%
Group2	3.15	100%	6.1	NA	3.9	100%	3.1	60%	0	0%
Group3	1.9	100%	4.6	NA	3.4	100%	3.7	100%	0	0%
Group4	2.7	100%	4.6	NA	3.5	100%	4.1	100%	NA	NA
Group5	4.32	100%	5.56	NA	6.8	100%	NA	NA	NA	NA
Group6	4.1	100%	5	NA	6.5	100%	NA	NA	NA	NA
Group7	0	0%			0	0%	0	0%	0	0%

* Mean for virus excretion includes only the data from birds which excreted measurable amount of virus.

All birds examined in 3 dpc are dead birds only * NA= Not Applicable

io.Figure 2. Percent of shedders birds after challenge

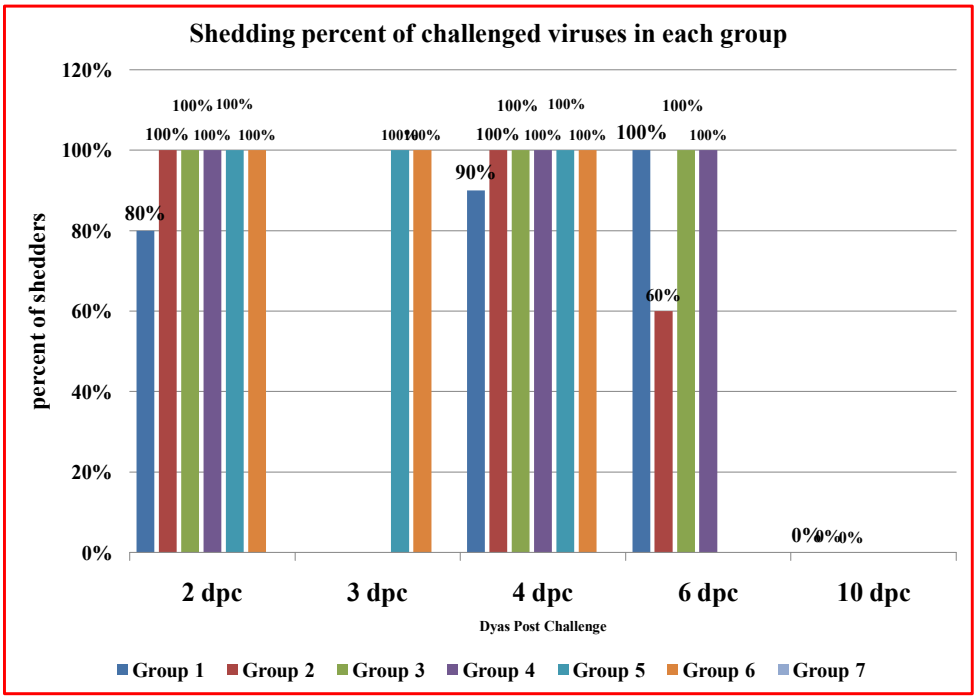
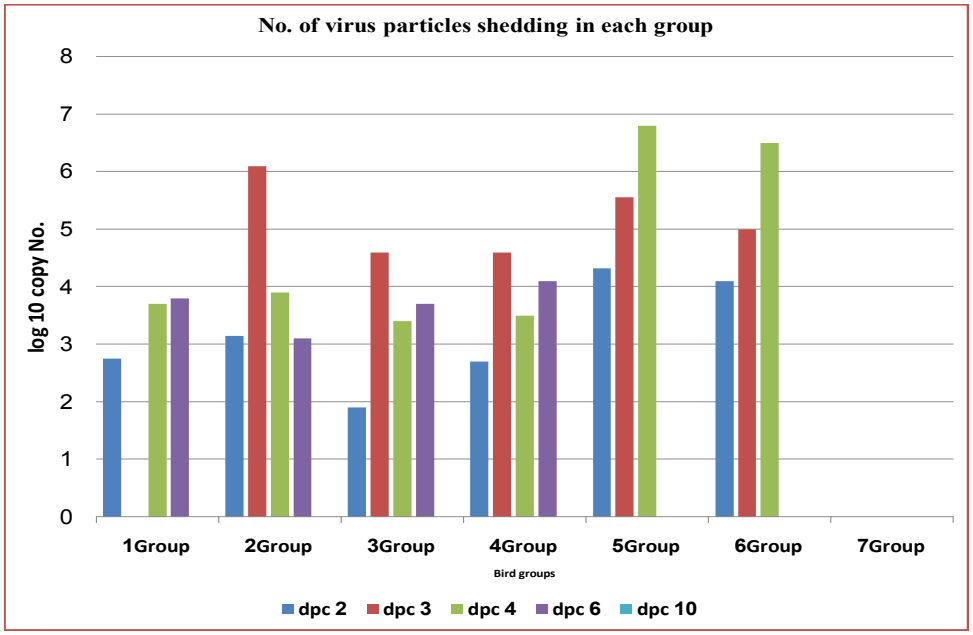


Figure 3. Amount of virus shedding log10 values after challenge.



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