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EVALUATION OF RECOMBINANT AVIAN INFLUENZA COMPARED TO INACTIVATED VACCINES TO INDUCE IMMUNE RESPONSE IN CHICKEN [52]

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ABSTRACT

The present Avian Influenza epidemic in Egypt is consider one of the major problems facing the poultry field and caused by circulation of geneticall-ly and antigenetically diverse influenza H5N1 viruses. This problem is controlled by applying vaccination. The objective was to determine the AI H5 recombinant vaccines efficacy (rHVT-H5, rFP-AI-H5 (Scotland and Ireland), k rND-AI and k rBuc-AI+ND) against classical and variant field HPAI H5N1 viruses in comparison to the traditionally inactivated whole AI virus vaccines as K R H5N1 / Egy, k H5N2 and k combined AI+ND vaccines. A single dose of the different types of vaccines either recombinant or inactivated whole virus vaccines was administered at different ages of chicken. Eight chicken groups were vaccinated with 8 vaccines and challenged after 4 weeks post vaccination to measure the protection %. Fecal and tracheal swabs were taken after 2 day post challenge to detect viral shedding. It was found that, live rFP-AI-H5 of both Scotland and Ireland strains induced poor clinical protection with high level of virus shedding. While, inactivated rND-AI, live rHVT-H5 and inactivated rBuc-AI+ND vaccines induced high protection rates ranged from 86.7% to 93.3% against both classical and variant HPAI viruses with a decrease or suppression of viruses shedding. In a parallel way, the inactivated whole virus AI vaccines either K R H5N1 / Egy, k H5N2 or k AI+ND induced a protection rates ranged from 85.7% to 100% with a high decrease in virus shedding levels. The data clearly indicate that inactivated whole AI virus and inactivated recombinant vaccines confers high levels of clinical protection with suppression in viral shedding compared to that of live recombinant vaccines except rHVT-H5 vaccine which induce a great level of protection and decrease in viral shedding in SPF chicken.

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INTRODUCTION

Influenza A viruses are enveloped, negative-strand RNA with a segmented genome, a member of the Orthomyxoviridae family **(Lupiani and Reddy 2009).** They infect a large variety of animal and birds species **(Munster and Fouchier 2009).** On the basis of the antigenic properties of their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Influenza A viruses are classified in birds into 16 HA (H1–16) and 9 NA (N1–9) subtypes, thus 144 possible combinations of which many could have been found in the field. **(Robert et al 2013).** The highly pathogenic avian influenza (HPAI) virus H5N1 causes multi-organ disease and death in poultry, resulting insignificant economic losses in the poultry industry. HPAI H5N1 also poses a major public health threat as it can be transmitted directly from infected poultry to humans with very high (60%) mortality rate. It is widely accepted that continued human exposure to influenza viruses circulating in wild and domestic avian species poses a permanent pandemic threat **(Yen and Webster 2009).**

Vaccination has been considered a suitable and powerful tool to support AI eradication **(Swayne 2003)**. Other vaccine strategies against HPAI H5N1 have been explored including live attenuated influenza vaccines **(Mueller et al 2010),** live vaccines based on heterologous viral vectors such as poxvirus **(Kreijtz, et al 2007),** adenovirus **(Gao, et al 2006)**, turkey herpesvirus (HVT) **(Gardin et al 2016)**, baculovirus **(Wu et al 2009)** and Newcastle disease virus **(DiNapoli et al 2010)**,

and DNA vaccination **(Rao et al 2008)**. While these different strategies often showed promising results, their applicability ultimately will depend on various important issues including safety, efficacy, production and costs **(Ellebedy and Webby 2009)**. An influenza vaccine based on recombinant purified HA could offer the following advantages: I) The HA antigen can be produced using safe, quality-controlled and scalable conditions. II) There will be no need for virus cultivation, thus avoiding the necessity a) to obtain viruses that replicate efficiently in eggs or cell culture, b) to use biocontainment facilities and c) to inactivate the virus using procedures that may affect antigenicity and raise safety concerns. III) There combinant HA protein can be highly purified thereby limiting adverse reactions caused e. g. by the presence of egg contaminants. IV) Immunization with recombinant HA will allow the serological differentiation of naturally infected from vaccinated animals/flocks (the so-called DIVA principle; **Van Oirschot, 2001)**. V) Recombinant HA vaccines are manufactured with a relatively short lead time, allowing an accelerated response to emerging influenza strains. Moreover, the disadvantages of some live recombinant vaccines include the risk of generating revertants and allow spread of genetically modified organisms in the environment **(Toro et al 2008)**. In Egypt, there are two conventional types of AI vaccines, the whole virus inactivated AI vaccines either reassortant H5N1 or LPAI H5N2 vaccines and recombinant live and dead vectored vaccines express AI-HA genes. This study aimed to determine the efficacy of the different types of recombinant AI vaccines and comparing the immune responses of the vaccines with that against inactivated AI vaccines and their ability to challenge the endemic Egyptian HPAI viruses.

MATERIALS AND METHODS

The experiments were done at Central Laboratory for Evaluation of Veterinary Biologics (CLEVB).

Animals

Eight groups of healthy chickens specific-pathogen-free (SPF) from Khom Oshem farm, El Fayoum, as one day and 4 week old. Each group of birds was housed separately in positive pressured isolators till used.

Recombinant & Inactivated Vaccines: were used in this study were tested in **Table (1).**

Table 1. Tested vaccine types, strains, routes and doses.

Vaccine types	AI Strains	Route & dose
Live recombinant fowl pox-AI (rFP + AI-H5 (Scotland)	A/chicken/Scotland/59	one day old chicks subcutanoesly 0.2ml/bird.
Live recombinant fowl pox-AI (rFP + AI-H5 (Ireland)	A/chicken/Irland/83	one day old chicks subcutanoesly 0.2ml/bird.
Recombinant turkey herpesvirus-AI (rHVT-H5)	A/swan/Hungary/4999/2006	one day old chicks subcutanoesly 0.2ml/bird.
Killed recombinant ND-AI (k rND-AI)	A/chicken/Egypt/1063/2010 LaSota	4 weeks old chicken subcutanoesly 0.5ml/bird.
Killed recombinant Baculo AI+ND (k rBuc-AI+ND)	A/duck/china/E319-2/2003 LaSota	4 weeks old chicken subcutanoesly 0.5ml/bird.
Killed Reassortant H5N1 (K R H5N1/Egy)	A/chicken/Egypt/A-18-H/09	4 weeks old chicken subcutanoesly 0.3ml/bird.
Killed H5N2 AI (k H5N2)	A/Chicken/Mexico/ 232/94/CPA	4 weeks old chicken subcutanoesly 0.5ml/bird.
Combined Killed AI H5N1 and Newcastle vaccine (K AI+ND)	A/Chicken/Mexico/232/94/CPA LaSota	4 weeks old chicken subcutanoesly 0.5ml/bird.

Antigen and antisera

The homologous AI antigens and antisera were obtained from the vaccine manufactures corresponding the vaccine type. While, ND Ag and antisera were obtained from GD, Netherland. The antigens were used for serological Heamagglutination Inhibition test.

Culture media for swabs processing

Tryptose phosphate broth code No. 0060-01 Difco laboratories, Detriot, Michigan, USA. It was used to cultivation of tracheal and cloacal swabs for determine of viral shedding.

Challenge virus:

- Variant AI strain: Local HPAI field isolate was isolated and identified by National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, (NLQP) as A/Ch/Egypt/1709-6/2008 (H5N1). Its titer was 10^{10} EID_{50}/ml. the challenge dose was adjusted to be 10^5 EID_{50}/0.1ml per bird and administrated intranasal.
- Classical AI strain: Local HPAI field isolate was obtained from Inactivated Viral Poultry Vaccines Department at Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) and identified by NLQP as A/Ch/Egypt/Qal-3/2016 (H5N1). Its titer was $10^{11.5}$ EID_{50}/ml. the challenge dose was adjusted to be $10^{7.5}$ EID_{50}/0.1ml per bird and administrated intranasal.

Potency test

50 SPF healthy chickens, one day and 4 week old are vaccinated S/C with one dose of each tested live recombinant and inactivated either recombinant or whole virus AI vaccines. Blood samples were drawn weekly post vaccination for serological analysis of AI immune response using heamagglutinin inhibition (HI) test. 30 birds out of each vaccinated and 20 birds of control groups were challenged with intranasaly both 10^5 EID_{50} challenge dose of variant HPAI and $10^{7.5}$ EID_{50} challenge dose of classical HPAI challenge viruses in 0.1ml/bird after 4 week old vaccination. The mortality and morbidity rates were recorded for each group during observation period (10 days) to measure the protection %.The assessment of viral shedding of HPAI challenge virus was performed through collection of orophayngeal swabs in tryptose media with antibiotic mixture on 2 days post challenge either from the vaccinated and control groups using virus reisolation in embryo chicken eggs (ECE) and rRT-PCR methods **(OIE, 2017)**.

Heamagglutinin inhibition assay (HI)

The Heamagglutinin inhibition (HI) test was performed as described previously **(OIE, 2017)** using 4HA units of the homologous AI antigen corresponding each vaccinal strains. The HI titers were determined as the reciprocal of the highest serum dilution in which inhibition of hemeagglutination was observed.

Determination of virus shedding

It was done according to **Pushko et al (2017)** in which the collected swabs after challenge on tryptose media were left at room temperature for 30 minutes then centrifuged at 3000xg for 10 minutes. The supernatant of each sample were diluted (2 fold) and each dilute was inoculated into five 9-day old- SPF ECE. The infected embryos were incubated at 37^{o}C for 5 days and examined daily. Alantoic fluid from each dead and live embryos were tested at the end of incubation period using slide Heamagglutinin (HA) test **(Swayne et al 1998)**.

Quantitative real-time RT-PCR (rRT-PCR)

For detection of viral shedding, orophayngeal swabs were processed for rRT-PCR according to **Das et al (2009)**. The viral RNA was extracted using RNA extraction kit (Qi Aamp viral RNA mini kit, Qiagen # 52904). Then the rRT-PCR was conducted according to the rRT-PCR kit instruction (Quanti Tech prabe RT-PCR, Qiagen #204443) using specific primer sets and probes as in **Table (2).**

Table 2. Oligonucleotide sequences of primer and probes for detection virus by rRT-PCR

Virus	Sequences		
AI (Hb)	Primers	H5LHI	ACATATGAC-TACCCACARTATTCAG
		H5RHI	AGACCAGCTAY-CATGATTGC
	Probe	H5PRO	(FAM)TCWACAGTGGCGAGT TCCCTAGCA(TAMRA)

The RT-PCR reaction scheme was on cycle at 50°C for 30 min, on cycle at 45°C for 15 min and 40 cycles (95°C for 10 seconds, 60°C for 1 min and 72°C for 10 seconds).

Experimental design

SPF chickens (430) were used for evaluation the efficacy of different recombinant and inactivated either recombinant or whole virus avian influenza vaccines. The chickens were divided into 8 experimental groups (50 birds /each), corresponding to each tested vaccine. Each group was divided into 3 subgroups, one had 20 birds for serological test and the 2nd, 3rd had 15 chickens/ each for challenge against classical (15 birds) and variant (15 birds) HPAI viruses. Also, the control non-vaccinated group (30 birds) was divided into 3 subgroups (10/each), the 1st and 2nd subgroup in challenged with classical and variant HPAI viruses respectively, while the 3rd subgroup kept as control unvaccinated and unchallenged.

RESULTS

Antibody response:

All chicken groups vaccinated with inactivated AI vaccines either whole virus or recombinant showed high antibody titer at 4 weeks post vaccination up to 7.1, 7, 8, 8,7.6 for k rND-AI, k r-baculo AI+ND, k R H5N1/Egy, k H5N2 and k AI+ND vaccines respectively. HI titers of chicken vaccinated with rHVT-H5 vaccines were much lower and did not reach to the basic level (7 log2) for vaccine release as shown in table (3). The HI titers increased for all groups until 10 WPV while control group show zero HI titer allover the weeks post vaccination.

Table 3. The mean of HI antibody titer in sera of chicken vaccinated with different types of AI vaccines using homologous antigens

Vaccine type	No. of Sample	Weeks post vaccination									
		1	2	3	4	5	6	7	8	9	10
rHVT-H5	10	2	3.6	4.5	5.6	6.1	6.4	7.1	7.3	7.5	7.8
k rND-AI	10	2.1	3.5	6.0	7.1	7.5	7.8	8.0	8.0	7.5	7.5
k rBuc-AI+ND	10	2.5	3.8	6.4	7.0	7.8	8.0	8.1	8.1	8.3	8.0
k R H5N1 / Egy	10	3.0	4.8	7.1	8	8	8	8	7.9	7.9	7.8
k H5N2	10	3.7	4.8	6.5	8	9.2	9.8	10.3	10.4	10.4	10.5
k AI+ND	10	2.5	4.3	6.4	7.6	8.5	8.7	8.7	8.9	9.0	9.0
Control	10	0	0	0	0	0	0	0	0	0	0

Vaccine efficacy

a) Protection %

The protection % of the tested AI vaccines was illustrated in **Tables (4 & 5).** The protection % of birds vaccinated with live rHVT-H5, rFP-AI-H5 (Scotland) and rFP-AI-H5 (Ireland) and challenged with local HPAI 2.2.1.1. Challenge virus were 93.3%, 20% and 33.3% respectively **(Table 4).** Moreover, The protection % of groups vaccinated with inactivated recombinant k rND-AI and k rBuc-AI+ND were 86.7% and 93.3%, while the protection % for the chicken groups vaccinated with the inactivated whole AI virus either K R H5N1 / Egy, K H5N2 and K AI+ND were 100%, 92.9% and 85.7% respectively. On the other hand, results of protection % of the vaccinated and control group, challenged with local HPAI 2.2.1.2 challenge virus were showed in table (5). The protection % of live rHVT-H5, rFP-AI-H5 (Scotland) and rFP-AI-H5 (Ireland) were 90%, 26.7% and 40% respectively, while the inactivated recombinant k rND-AI and k rBuc-AI+ND protect the chicken against challenge virus with a ratio of 86.7% and 93.3%. Also, the chicken vaccinated with inactivated k R H5N1 /Egy, k H5N2 and k AI+ND were protected with a percentage reach to 100%, 93.3% and 86.7% respectively.

Table 4. The protection % of different types AI vaccines against the challenge with variant strain of Egyptian HPAI H5N1 virus.

Vaccine type	No. of birds /challenge	Challenge (4 weeks post vaccination) Days post challenge (DPC)										Total deaths	Protection %
		1	2	3	4	5	6	7	8	9	10		
rFP-AI-H5 (Scotland)	15			7	4	1						12/15	20%
rFP-AI-H5 (Ireland)	15			5	4	1						10/15	33.3%
rHVT-H5	15				1							1/15	93.3%
k rND-AI	15			1		1						2/15	86.7%
k rBuc-AI+ND	15					1						1/15	93.3%
K R H5N1 / Egy	15											0/15	100%
K H5N2	15	1			1							1/14	92.9%
K AI+ND	15	1			1	1						2/14	85.7%
Control	10		1	9								10/10	0

Table 5. The protection % of different types AI vaccines against the challenge with classical strain of Egyptian HPAI H5N1 virus.

Vaccine type	No. of birds /challenge	Challenge (4 weeks post vaccination) Days post challenge (DPC)										Total deaths	Protection %
		1	2	3	4	5	6	7	8	9	10		
rFP-AI-H5 (Scotland)	15			6	4	1						11/15	26.7%
rFP-AI-H5 (Ireland)	15			5	3	1						9/15	40%
rHVT-H5	15				1							1/15	93.3%
k rND-AI	15					1	1					2/15	86.7%
k rBuc-AI+ND	15						1					1/15	93.3%
k R H5N1 / Egy	15											0/15	100%
k H5N2	15				1							1/15	93.3%
k AI+ND	15				2							2/15	86.7%
Control	10		1	8	1							10/10	0

b) Determination of virus shedding

1 Virus reisolation

The virus shedding was determined using virus reisolation in SPF ECE from tracheal and fecal swabs from both vaccinated and control groups on day 2 post challenge. All tracheal and cloacal swabs collected from birds in the control groups challenged with both local variant 2.2.1.1 and classical 2.2.1.2 HPAI virus were positive for virus reisolation as shown in **Tables (6 & 7).** Results for vaccinated birds challenged with the variant 2.2.1.1 HPAI virus are shown in table (6). It was observed that in case of live rFP-AI-H5 (Scotland) and (Ireland) there was very low level of challenge virus reduction ranged from $10^{0.3}$-$10^{0.7}$ and 11-13/15 cloacal and tracheal swabs were positive for virus reisolation, but the virus shedding of k rND-AI and k rBuc-AI+ND was reduced with a level ranged 3-3.2 respectively, while the rHVT-H5 vaccine evoked a reduction in the challenge virus dose shed from respiratory tract equal $10^{3.4}$ and no viral shedding from digestive tract. Also, it was showed that there was reduction in variant challenge virus replication either 2.5, 3 and 3.5 EID_{50} from tracheal swabs of chicken vaccinated with inactivated whole virus either k AI+ND, k R H5N1 /Egy and k H5N2 vaccines respectively, in addition, to a very low number of cloacal swabs were positive for virus reisolation (1/15, 2/15 and 0/15). The results of virus shedding from the vaccinated birds challenged with classical 2.2.1.2 HPAI virus were cleared in **Table (7).** It was observed that there was a reduction in the viral shedding from tracheal swabs with levels of 0.5, 0.7, 4.8, 3.5, 6, 4.5, 4 & 6 EID_{50} for groups vaccinated with rHVT-H5, rFP-AI-H5 (Scotland), rFP-AI-H5 (Ireland), k rND-AI and k rBuc-AI+ND, k R H5N1 /Egy, k H5N2 and k AI+ND vaccines respectively. In addition to there were

very high levels of viral shedding from the fecal swabs of chicken vaccinated with rFP-AI-H5 of both types were 13/15 bird were positive; while the viral shedding from cloaca of the bird vaccinated with other type of AI vaccines ranged from 0/15 (in case of rHVT-H5, k R H5N1 /Egy and k H5N2) to 1/15, 1/15 & 3/15 in case of k rBuc-AI+ND, k AI+ND and k rND-AI vaccines, respectively.

2 rRT-PCR

All the tracheal & fecal swabs taken from the vaccinated & control groups after challenge with the variant HPAI virus were tested for viral shedding using rRT-PCR as shown in **Tables (6 & 7).** It was cleared that the chicken vaccinated with two kinds of rFP-AI vaccines shed a high level (1.1 & $1.2X10^6$) of challenge virus with low CT (26) as the control groups ($2.7X10^6$) either from respiratory and digestive tracts. The results clearly showed that birds group vaccinated k R H5N1 /Egy vaccine shed a relatively lower amount of challenge virus ($1.1X10^4$) from the respiratory tract while their fecal swabs were negative. The number of virus copies shed by chicken groups vaccinated with rHVT-H5, k AI+ND & k rBuc-AI+ND vaccines were $2.794X10^4$, $4.001X10^4$ & $1.694X10^4$ respectively, and their fecal swabs were negative. Moreover, the amount of virus shed by both groups vaccinated with k rND-AI & k AI+ND were $4.338X10^5$ & $1.122X10^5$ with no viral shedding from their cloaca. On the other hand, the viral shedding from the vaccinated birds challenged with classical strain was showed in **Table (7)** with the same manner there was high level of viral shedding from the group vaccinated with the rFP-AI-H5 of both types ($1.3X10^6$) and showed positive fecal swabs in parallel to the control group which show positive fecal swabs and high titer of viral shedding from tracheal swabs ($1.6X10^6$). moreover, the viral shedding from tracheal swabs showed increased level from $1.2X10^4$, $1.58X10^4$, $2.6X10^4$, $3.9X10^4$, $4X10^5$ & $1.1X10^5$ in case of k R H5N1/Egy**,** k rBuc-AI+ND, rHVT-H5, k AI+ND**,** k rND-AI & k AI+ND vaccines respectively with a negative results in their fecal swabs as detected with rRT-PCR.

Table 6. Viral shedding from vaccinated chicken with different AI vaccine types and challenged with variant strain (2008) of Egyptian HPAI H5N1 virus.

vaccine	Protection %	Challenge (4 WPV)						
		Viral shedding 2 days post challenge (DPC) by ECE				Viral shedding 2 DPC by r-RT-PCR		
		Tracheal swab			Fecal swab (+ve/total)	Tracheal swab		Fecal swab (+ve/total)
		Sample (+ve/total)	Virus titer EID_{50}/log_{10}	Viral reduction Log_{10}		CT	Conc. (copies/140ml)	
rFP-AI-H5 (Scotland)	20%	13/15	5.2	0.3	13/15	26.84	1.1×10^6	+ve
rFP-AI-H5 (Ireland)	33.3%	13/15	4.8	0.7	11/15	26.94	1.2×10^6	+ve
rHVT-H5	93.3%	1/15	2.2	3.3	0/15	32.63	2.794×10^4	-ve
k rND-AI	86.7%	5/15	3.3	2.2	2/15	28.56	4.338×10^5	-ve
k rBuc-AI+ND	93.3%	3/15	2.5	3	0/15	32.94	1.694×10^4	-ve
k R H5N1 / Egy	100%	1/15	2	3.5	0/15	33.44	1.108×10^4	-ve
k H5N2	92.9%	2/15	2.5	3	1/15	32.02	4.001×10^4	-ve
k AI+ND	85.7%	4/15	3	2.5	2/15	30.73	1.122×10^5	-ve
Control	0	5/5	5.5	0	5/5	25.63	2.7×10^6	+ve

Table 7. Viral shedding from vaccinated chicken with different AI vaccine types and challenged with classical strain (2016) of Egyptian HPAI H5N1 virus.

Vaccine	Protection %	Challenge (4 WPV)						
		Viral shedding 2 days post challenge (DPC) by ECE				Viral shedding 2 DPC by r-RT-PCR		
		Tracheal swab			Fecal swab (+ve/total)	Tracheal swab		Fecal swab (+ve/total)
		Sample (+ve/total)	Virus titer EID_{50}/ log_{10}	Viral reduction Log_{10}		CT	Conc. (copies/140ml)	
rFP-AI-H5 (Scotland)	26.7%	13/15	8	0.5	12/15	27.10	1.3×10^6	+ve
rFP-AI-H5 (Ireland)	40%	12/15	7.8	0.7	12/15	27.26	1.338×10^6	+ve
rHVT-H5	93.3%	1/15	2.5	6	0/15	32.75	2.675×10^4	-ve
k rND-AI	86.7%	6/15	5	3.5	3/15	28.69	4.001×10^5	-ve
k rBuc-AI+ND	93.3%	4/15	3.7	4.8	1/15	33.02	1.586×10^4	-ve
k R H5N1 / Egy	100%	1/15	2.5	6	0/15	33.33	1.22×10^4	-ve
k H5N2	93.3%	2/15	4	4.5	0/15	32.24	3.964×10^4	-ve
k AI+ND	86.7%	3/15	4.5	4	1/15	30.75	1.108×10^5	-ve
Control	0	5/5	8.5	0	5/5	26.48	1.6×10^6	+ve

DISCUSSION

The ongoing H5N1 HPAI virus epidemic in poultry in Egypt underlines difficulties in controlling and eradicated in this infection vaccination was introduced in Egypt as a common tool to prevent or reduce loss due to AI infection. Although the efforts in controlling the infection, the virus is still circulating causing economic losses to the poultry sector with direct and indirect pressure on livehood of people. A variety of vaccines are used to control the disease in chicken as inactivated whole AI virus oil emulsion vaccines. Other types of live and inactivated vectored vaccines have been developed for AI vaccination protocol as fowl pox **(Beard et al (1991)**, baculovirus **(Yang et al 2007)**, herpes **(Gardin et al 2016)** or Newcastle dis. Virus **(Ge et al 2007)**.

In the present study, not all tested AI vaccines induced substantial HI antibody responses in vaccinated chicken (table 3). The inactivated whole virus mono AI vaccines either K R H5N1 / Egy or K H5N2 induced a homogenous HI antibody response against the homologous antigens allover period of observation (10 WPV), while, the live vector recombinant vaccine (rHVT-H5) was lower immunogenic in a HI antibody response against the corresponding virus **(Tian et al 2005).** Also, the inactivated recombinant vaccines (k rND-AI and k rBuc-AI+ND) showed increase in antibody response mimic the inactivated vaccines. In endemic H5 HPAI virus countries such as Egypt, the frequent antigenic drift of AIV requires continuous and strict vaccine evaluation using challenge test to keep up the vaccine efficacy **(Suarez, 2010).** In the present study, the efficacy of different recombinant AI vaccines against both clade 2.2.1.2 (classical group) and 2.2.1.1 (variant group) of H5N1 HPAI viruses in comparison with the inactivated oil emulsion AI vaccines. Representatives of dominant clades of the AI viruses were selected based on the year of isolation, the pathogenicity and genetic characterization. The two challenge virus were A/Ch/Egypt/1709-6/2008 (clade 2.2.1.1) representative as variant strain and A/Ch/Egypt/Qal-3/2016 (clade 2.2.1.2) which representative as classical strain. The single-shot vaccination scheme of the tested recombinant and oil emulsion AI vaccines was done. The vaccine efficacy of the tested vaccines depend on that they provide complete protection from morbidity, mortality and virus shedding against a lethal dose of challenge viruses **(Kim et al 2008).** The protection of chicken was evaluated by prevention of respiratory or general clinical signs (morbidity), deaths (mortality) and virus shedding from tracheal during observation period after challenge **(Xie and Stone, 1990).**

AI virus is usually isolated and detected by inoculation of swabs from tracheal or cloacal pathways of infected birds into chorioallantoic sac of embryonating chicken egg **(Nayak et al 2009)**. rRT-PCR is a relatively new technology that has been used for AI virus detection since the early 2000s **(Pushko et al 2017)**. In this study, the viral

shedding was detected from tracheal swabs of the challenged birds by both viruses reisolation in ECE and rRT-PCR. All the chickens immunized with two types of rFP-AI-H5 Scotland and Ireland were not protected against both the classical (20 & 33.3%, respectively) and variant (26 & 40%, respectively) challenge viruses **(Tables 4 & 5).** These results are matching with **Swayne et al (2000)** who reported the effectiveness of recombinant hatchery vaccine using rFP-AI-H5 due to prior exposure or vaccination of breeder with fowl pox virus alone leading to vaccination failure. Also, from **Tables (4 & 5)** the present study showed that there was greater viral shedding of HPAI challenge viruses (classical & variant strains) from tracheal swabs in case of chicken vaccinated with two types of rFP-AI-H5 when estimated either by viral reisolation in ECE or rRT-PCR methods. Also, the tracheal swabs from the some birds were positive for viral shedding of the two challenge viruses when examined by rRT-PCR. These data are consistent with the studies carried out by **Lee and Suarez, (2004)** who found that there was a correlation between haemagglutinin sequence similarity and the ability of vaccine to reduce the tracheal titers of challenge virus.

The k rBuc-AI+ND vaccine the protection % was 93.3% against the two types of challenge virus as shown in **Tables (4 & 5).** Also, it was observed that there was a great reduction in viral shedding from the vaccinated birds with a ratio of 3 $\log_{10}$ and 4.8 $\log_{10}$ after challenge with variant and classical challenge viruses, respectively when detected with egg inoculation system **(Tables 6 & 7).** The tracheal swabs taken from vaccinated-challenged birds against two challenge viruses were negative when examined by rRT-PCR. Previous studies showed that full protection was occur following vaccination of chickens with one shot of k rBuc-AI+ND vaccine against challenge with HPAIVs **(Beato, et al 2013**). Another study by **Crawford et al (1999)** demonstrated protection of the vaccinated bird with baculovirus based vaccines against heterologous challenge viruses. Also, **Tables (4 & 5)** gives a simple overview of the protection results collected through challenge tests conducted with rHVT-H5 vaccine against classical and variant HPAI infection. The protection % of chicken vaccinated with one shot of rHVT-H5 at one day old and challenged at 4 WPV was 93.3% against HPAIV strains of different clades of H5N1 and different doses of challenge viruses. A reduction of virus shedding, both by ECE virus reisolation and rRT-PCR, was done following challenge by classical and variant viruses. It was showed that from **Tables (6 & 7)** there was a reduction in viral shedding within the acceptable ranges (ECE and rRT-PCR) against classical and variant viruses, this agreed with **Kilany et al (2014)** who said that the higher homology between the H5 gene in the commercially used AI vaccine induced in the rHVT-H5 and the circulated viruses, the higher protection afforded from this vaccines. The studies indicated that rHVT-H5 has no interference with MDA and therefore low effect in level of clinical protection. The rHVT-H5 vaccine has also been studied in short cycle birds (broiler) and its efficacy confirmed with a protection range of 90-100% depending on the presence or absence of MDA **(Ranw et al (2012)** also showed 95% clinical protection in broiler birds vaccinated with rHVT-H5 at one day old. **Kilany et al (2014)** observed that single hatchery vaccination with rHVT-H5 vaccine confers protection against HPAI H5 in commercial layer chickens at least during rearing period (19 wks).

However, the k rND-AI vaccine provided 86.7% protection for both challenge viruses (variant & classical) as shown in **Tables (4 & 5).** Reduction of virus shedding detection, both by two methods either virus reisolation ECE as well as rRT-PCR shown in **Tables (6 & 7).** It was observed that the vaccine was reduce the viral shedding in vaccinated birds which challenged with variant HPAI virus with a range of 2.2 EID_{50} and $4.3X10^5$ RNA copies when compared with the control birds. However, the reduction in the viral shedding in vaccinated chicken challenged with classical HPAI virus was ranged from 3.5 EID_{50} and $4X10^5$ RNA copies. In studies by **Nayak et al (2009)**, recombinant NDV viruses expressing the HA gene of H5N1 AIV that were constructed using the LaSota strain induced significant HI antibody responses against NDV and H5N1 AIV and provided complete immune protection from challenges with NDV as well as from lethal challenges with both homologous and heterologous H5N1 AIV.

Meanwhile, the protection % of chicken vaccinated with one dose of K R H5N1 / Egy, K H5N2 and K H5N2 + ND vaccines were 100%, 92.9% and 85.7% respectively (table 4) after challenge with HPAI variant strain, but from the data shown in **Table (5),** it is observed that K R H5N1 / Egy, K H5N2 and K H5N2 + ND vaccines could protect the chicken against classical strain of HPAI with 100%, 93.3% and 86.7%, respectively. Reduction of virus shedding detection, both by two methods either virus reisolation ECE as well as rRT-PCR was always done 2 days following challenge either

by classical and variant HPAI strains **(Tables 6 & 7).** It was observed that the K R H5N1 / Egy, K H5N2 and K H5N2 + ND vaccines were reduce the viral shedding in vaccinated birds which challenged with variant HPAI virus with a range of 3.5-2.5 EID_{50} and $1.2X10^3$ RNA copies when compared with the control birds. However, the reduction in the viral shedding in vaccinated chicken challenged with classical HPAI virus was ranged from 4-6 EID_{50} and $1.2X10^3$ RNA copies.

The results demonstrated that inactivated vaccines (either whole virus or recombinant) and live rHVT-H5 vaccines induced more effective protection against both classical and variant HPAIV challenge viruses than live rFP-AI-H5 vaccines. Some avian viruses have been used as viral vectors to develop recombinant AI vaccines as rFPV (**Swayne et al 2000**) or ND (**Nayak et al 2009**) expressing AIV H5 gene.

But, it was observed that the live rHVT-AI vaccine induced more effective and prolonged protection against HPAI challenge viruses than the live rFP-AI-H5 vaccines and this agreed with **(Li et al 2011)** who found that chicken vaccinated with rHVT vaccine expressing AIH5 HA were protected against AI infection. One of the concerns of vaccination against AI is that single dose of current vaccines do not produce sufficient immunity to completely prevent AI infection and subsequent virus transmission. Although, our study demonstrated that vaccination with inactivated AI vaccines (either whole virus or recombinant) able to reduce the spread of AIV within flock **(Swayne, 2015)** and reduce uniform protection from the clinical signs or death after challenge by different strains of HPAI viruses.

Finally, the data from this study cleared that the using of killed AI vaccines induced higher HI antibody titer, protection and reduction in viral shedding of challenge virus more than the live vector recombinant vaccines. Also, the rHVT-AI vaccine is more effective than other live recombinant AI vaccines due to HVT behavior in induction of prolonged immune response **(Kapczynski et al 2012).** Also, the usage of killed recombinant AI vaccines can be more effective for allowing differentiation of infected from vaccinated birds (DIVA) **(Lozano-Dubernard et al 2010)** beside their ability for induction of good immunity and reducing high level of viral shedding **(Cui et al 2013).** On the other hand, our study illustrated that all inactivated AI vaccines (whole virus or recombinant) and rHVT-AI vaccines are able to produce high protective immunity with good reduction in viral shedding against both classical and variant strains of HPAI virus a proximately with the same levels. These agreed with the **Kim et al (2008)** who said that the good manufacturing vaccines can protect chicken against different strains of AIV. So, there are other parameters which increase vaccine efficacy as good manufacturing procedures, proper adjuvant system, biosafety and biosecurity facilities and route of immunization **(OIE, 2017).**

However, all these AI vaccines have advantages and disadvantages, a spectrum of effective vaccines is highly desirable and the licensing of available vaccines should be promoted to supplement and expand current intervention strategies against avian influenza consistent under different epidemiological situations.

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