Marden *et al., J. of Virol. Sci.,* Vol. 4: 57-71, 2018
ISSN: 1685-1687

Journal of Virological Sciences

RESEARCH

Comparative Studies on Evaluation of Different Avian Influenza Inactivated Vaccines under Local Conditions in Egypt

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ABSTRACT

Background: Vaccination strategies have an important role in the limitation of viral infection and spread to the surrounding environment. The evaluation methods of the AI vaccines are very important for controlling the license of the bad vaccines. Comparative studies between the invitro and invivo methods for 8 types of AI vaccines (5 batches for each) to evaluate their efficacies were done.

Methods: The invivo method, was done through the vaccination-challenge pathway of SPF chickens to estimate the protection percent of each vaccine against both classical and variant HPAI viruses. While, the invitro method was done by the direct detection of the viral content and viral identity using the rRT-PCR technique from final product of these inactivated vaccines.

Results: The data of this study showed that sufficient HA antigen content and similar HA sequences with the field HPAI challenge viruses are needed to produce a consistent and high protection percent, but, they are not enough to assess the vaccine efficacy.

Conclusion: The invitro assessment of protection was not considered an alternative methods to the direct assessment of vaccine efficacy (vaccination-challenge), but it was used as complementary steps for vaccine evaluation protocol.

Keywords: avian influenza, inactivated vaccines, protection percent, vaccine viral strain and vaccine viral

BACKGROUND:

Since the Highly pathogenic avian influenza virus H5N1 was reported in Egypt in 2006 it spread widely in many governorates within poultry populations and many other species of birds, animals and also human. The devastating disease becomes endemic since 2008 causing great economic losses in the Egyptian poultry industry and also causing many human fatal cases (Awad *et al.,* 2015 and Hussein *et al.,* 2015).

The AI virus evolves through its spread journey leading to emergence of variant strains which firstly appeared in late 2007 (2007/2008 season) and forming a variant group (2.2.1.1) of the Egyptian subclade. Also another variant one (2.2.1.1.a.) appeared in 2008. These variant groups have many mutations in the antigenic sites of their HA antigen making them different from the original classical group (2.2.1) which also evolved with a divergence of a new classical subgroup (2.2.1.2) appeared in 2008. This new phenotypic subgroup still persistent and predominant from 2011/2012 season up till now with nearly disappearance of the variant linage (Abdelwhab *et al,.* 2012; El-Zoghby *et al.,* 2012; WHO/ OIE/FAO, 2012 ; Ibrahim *et al.,* 2013; WHO/ OIE/ FAO, 2014; Arafa *et al.,* 2015; Hussein *et al.,* 2015 and Arafa *et al.,* 2016).

Although many control measures like culling of infected poultry, zoning and movement restrictions, and vaccination were applied in Egypt to restrict the spread of infection they were with limited effect and their application were stopped except vaccination which still the main control measure applied up till now (Peyre *et al.,* 2009 and Kayali *et al.,* 2016).

The inactivated vaccines are considered the main type of vaccines used in Egypt against AIV. These vaccines differ from each other in their viral content, the viral strain used in their preparation and the quality of their processing which reflect directly on the yielded protection

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percent and viral shedding following challenge with the local virulent AI virus (Hafez *et al.*, 2010 and Kayali *et al.,* 2016).

In this study we aim to: I) apply of an alternative method for direct vaccine strain identity and quantification by rtRT-PCR in inactivated vaccines. II) Do Comparative studies on the efficacy of different imported and local inactivated AI vaccines through: 1) Determination of relationship between the type of local HPAI H5N1 challenge strain (classical and variant) and protection %. 2) Determination of relationship between vaccine strain similarity to challenge strains and protection %. 3) Determination of relationship between vaccine antigen content and protection %.

MATERIALS AND METHODS

1. Vaccines:

Different inactivated AI vaccines with different vaccine strains from many manufacturers were tested in this study as shown in table (1) as follow:

Table (1): Types of inactivated AI vaccines (strains, routs and doses).

S	Vaccine type	Vaccine strain	Vaccine dose	Vaccination rout	Producer
1	Inactivated H5N1 Egy/PR8-1 **(kH5N1/Egy)**	Reassortant with its HA and NA genes were derived from Ck/Egypt/A-18/09 (H5N1)	0.3 ml	subcutaneously at the lower third of the neck	Harbin Veterinary Research Institute (HVRI), China
2	Inactivated H5N1 /Re-1 **(kH5N1/Re-1)**	Reassortant its HA and NA genes were derived from A/Goose/Guangdong/96 (H5N1)	0.3 ml		Zhaoqing Dahuanong Biology Medicine Co. Ltd., China
3	Inactivated H5N1/local 1 **(kH5N1/loc 1)**	rgA/Duck/Egypt/M2583D/2010(H5N1) rgA/Chicken/Egypt/Q1995D/2010(H5N1)	0.5 ml		Vaccine and Serum Veterinary Research Institute (VSVRI), Egypt
4	Inactivated H5N1 /local 2 **(kH5N1/loc 2)**	rg A/Duck/Egypt/M2583D/2010 (H5N1) rgA/Chicken/Egypt/Q1995D/2010(H5N1)	0.5 ml		Middle East for Veterinary Vaccine campany (ME VAC), Egypt
5	Inactivated H5N2 /Mexican 1 **(kH5N2/Mex 1)**	A/Chicken/Mexico/232/94 /CPA (H5N2) LPAI	0.5 ml		Boehringer ingelheim Vetemedica, Mexico
6	Inactivated H5N2 /Mexican 2 **(kH5N2/Mex 2)**	A/Chicken/Mexico/232/94 /CPA (H5N2) LPAI	0.5 ml		Ceva Kemia for Biomune, Mexico
7	Inactivated H5N2 /Potsdam **(kH5N2/Pot)**	A/Duck/Potsdam/1402/86 (H5N2) LPAI	0.5 ml		Intervet International B.V.-Boxmeer – Holland
8	Inactivated H5N3**(kH5N3)**	Reassortant AIV H5N3 2228-11JAN05 with its HA gene was derived from rgA/Chicken/Vietnam/C58/2004(Δ2005) and NA gene was derived from A/Duck/Germany/1215/73	0.5 ml		Fort Dodge Animal Health, USA

*N.B.:- 5 batches from each vaccine type were used in the experiment.

2. Experimental host:

a) SPF chickens:

A total of 2030 SPF (specific pathogen free) chickens were obtained as one day old chicks from Kom Oshim for SPF ECEs farm, El-Fayoum, Egypt. They were used for evaluation of the tested inactivated AI vaccines.

b) SPF ECEs:

SPF ECEs (embryonated chicken eggs), 9 days old, were obtained from Kom Oshim for SPF ECEs farm, El-Fayoum, Egypt. They were used for preparation of challenge viruses.

3. Antigens and antisera:

The standard homologous AI antigens and antisera were obtained from each AI vaccine manufacturer corresponding to the vaccine strain of each vaccine type. Also standard antigens and antisera for NDV, EDS and H9N2 were obtained (Animal Health Service Deventer "GD", Netherland). They were used in HA and HI tests.

4. Challenge viruses:

Two Local HPAI Egyptian field isolates were used as challenge viruses as follow:

a) 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.

b) Classical AI strain:

Local HPAI field isolate was isolated in 2016 from Qaluobia governorate by Inactivated Viral Poultry Vaccines Evaluation Department at Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) and identified by NLQP as A/Ch/Egypt/Qal-3/2016 (H5N1), accession # MF664437. 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.

5. *Invitro* evaluation of AI vaccines:

a. Antigen recovery from the inactivated AI vaccines:

The water phase of the tested inactivated AI vaccines was separated from the oil phase by Isopropyl Myrstate (IPM) **(Roth, Art.5527.1)** according to **Maas *et al.*, (2003).**

b. Antigen quantification:

The eluted antigens were quantified using HA test according to **(OIE, 2017)**. A reference antigen and a negative control one were included on each plate.

c. Identification of AI vaccine strains:

Direct detection of the vaccine strain type and titer of different AI vaccines was done using rRT-PCR technique as explained by Swayne *et al.*, (2006.).The procedure was done as follow:

I. Extraction of viral antigens from oil emulsion vaccines by trizol LS (Invitrogen) refer to the standard operating procedure provided by Swayne *et al.*, (2006.).

II. rRT-PCR test for typing of the vaccine strains were done according to Das *et al.*, (2006).

The viral RNA was extracted using (QIAamp Viral RNA extraction kit, QIAGEN Gmb H, Germany, catalogue No. 52904). Then rRT-PCR was conducted according the rRT-PCR kit instruction (QuantiTect probe RT-PCR (QIAGEN) catalogue No. 204443). Using specific primer

sets and probe as in table (2). The rRT-PCR reaction scheme was one cycle at 50 ºC for 30 minutes, one cycle at 95 ºC for 15 minutes, and 40 cycles (95 ºC for10 seconds, 54 ºC for 30 seconds and 72 ºC for 10 seconds).

Table (2): primer and probe sequences used in rRT-PCR technique.

Gene	Primer /probe	Primer & probe sequence	Ref.
H5 gene (AIV)	F-H5	5'-ACA TAT GAG TAC CCA CAT TAT TCA G-3'	**LÖndt *et al.*, (2008)**
	R-H5	5'-AGA CCA GCT ATC ATG ATT GC-3'	
	H5 Probe	[FAM] 5'-TCA CAG GGC GAG TTC CCT AGC A-3' [TAMRA]	

d. Sequence analysis of AI vaccine strains and challenge viruses:

The sequence analysis and comparison between sequences of the locally isolated challenge viruses (either classical strain under accession number# MF664437 and variant strain under accession number # EU 717857 on Genbank) and the sequences posted in gene bank for vaccine strains of the tested AI vaccines were done using Blast program (Altschul *et al.,* 1990).

***Invivo* evaluation of AI vaccines:**

e. Identity test:

The identity of AI antigen type incorporated in the inactivated vaccines under test is carried out through testing the sera collected from vaccinated chicken (in conjunction with potency test) by HI test. This was carried according to (Egyptian Standard Regulations for Evaluation of Veterinary Biologics, 2009).

f. Potency test:

It include the evaluation of the vaccine AI strain efficacy of different tested AI vaccines against Egyptian classical and variant isolates using vaccination-challenge test (Kapczynski *et al. 2015*). SPF chickens, 4 weeks old, were vaccinated subcutaneously with field dose recommended by the tested vaccines producers. Blood samples were taken weekly till 4th week post vaccination and serum samples were separated and inactivated at 56 ºC for 30 minutes. The antibody level of each vaccine was determined by HI test using homologous and heterologous antigens. The efficacy of each tested vaccine was done by challenging the vaccinated chickens at 4 weeks post vaccination against both classical and variant local HPAI viruses and observed for 10 days post challenge. The challenge dose was adjusted to be 10^5 EID_{50} for variant strain and $10^{7.5}$ EID_{50} for classical strain according to their pathogenicityies in chicken and administrated as 0.1 ml intranasally. Groups of control SPF chickens were infected with the same doses of the challenge viruses. All the dead and clinically infected birds were recorded during observation period for detection of the protection percent of each tested vaccine.

6. Experimental design:

2030 SPF chickens were used for efficacy evaluation of different tested inactivated AIV vaccines. The chickens were divided into 40 experimental groups (50 birds/each), corresponding to each tested vaccine batch (5 batches/ vaccine type). Each was divided into 3 subgroups, the 1st subgroup had 20 birds for challenge test with classical viral strain at 4 WPV, the 2nd subgroup had 20 birds for challenge test with variant viral strain at 4 WPV and the 3rd subgroup had 10 for serological analysis 4 WPV. Also the control non-vaccinated group (30 birds) was divided into 3 subgroups (10 birds each) as control subgroups for the same subgroups of the tested vaccines.

RESULTS

I- *Invitro* evaluation of AI vaccines:

A. Results of *invitro* vaccine strain identification:

The invitro identification of the vaccine strains of the tested inactivated vaccines by rRT-PCR using specific primers pairs towards AIV (H5, H9 genes) and NDV. The results approved that all vaccines were AI H5 positive and (H9 and NDV) negative.

B. Results of antigen content:

The antigen content of different tested inactivated AI vaccines was detected invitro by estimating HA and rRT-PCR titers as shown in table (3). It was observed that HA titers of kH5N1/Egy, kH5N1/Re-1, kH5N1/loc 1, kH5N1/loc 2, kH5N2/Mex 1, kH5N2/Mex 2, kH5N2/Pot and kH5N3 were ≥ 9, 8, 8, 7, 8, 8, 8 and 6 log2 respectively as found in their scientific files before inactivation and became 7.6, 7, 7.4, 6.4, 7.2, 6.6, 6.6 and 6 log2 when *invitro* measured in final product of these vaccines respectively. Also, the viral titer of the same tested vaccine were ranged from ≥ 8.5, 8.5, 9, 8.5, 8.5, 8, 8 and 7 EID_{50}/ml before inactivation as mentioned in their scientific files and were 1.202 x 10^5, 3.632 x 10^4, 6.505 x 10^5, 2.884 x 10^4, 5.311 x 10^4, 2.813 x 10^4, 1.595 x 10^4 and 1.484 x 10^4 copies/140 µl when measured *invitro* by rRT-PCR respectively. These vaccines induced HI titers at the 4th WPV 8.8, 8, 8.2 "7.4", 7.8 "7.1", 11.3, 7.8, 7.4 and 8.5 log2 respectively.

Table (3): Results of viral content for different AI vaccines.

Vaccine	No. of batches	Viral content before inactivation *		*Invitro* viral content in final product		*Invivo* mean HI titer at 4th WPV **
		HA titer (log2)	EID_{50} (log10) /ml	HA titer (log2)	copies/140 µl by rtRT-PCR	
kH5N1/Egy	5	≥ 9	≥ 8.5	7.6	1.202 x 10^5	8.8
kH5N1/Re-1	5	≥ 8	≥ 8.5	7	3.632 x 10^4	8
kH5N1/loc 1^	5	≥ 8	≥ 9	7.4	6.505 x 10^5	8.2 "7.4"
kH5N1/loc 2^	5	≥ 7	≥ 8.5	6.4	2.884 x 10^4	7.8 "7.1"
kH5N2/Mex 1	5	≥ 8	≥ 8.5	7.2	5.311 x 10^4	11.3
kH5N2/Mex 2	5	≥ 8	≥ 8	6.6	2.813 x 10^4	7.8
kH5N2/Pot	5	≥ 8	≥ 8	6.6	1.595 x 10^4	7.4
kH5N3	5	≥ 6	≥ 7	6	1.484 x 10^4	8.5

*data before inactivation were obtained from the scientific files.

** WPV: week post vaccination.

^: HI results for these types of vaccines were divided into 2 results the 1st results were the HI results of sera against Q-Ag (rg A/Chicken/Egypt/ Q1995D/2010 (H5N1)) while the 2nd results were the HI results of sera against M-Ag (rg A/Duck/Egypt/M2583D/2010 (H5N1)).

C. Results of AI vaccine strains similarities to the challenge viruses:

From table (4) it was found that, the vaccine strains of the tested inactivated vaccines kH5N1/Egy, kH5N1/Re-1, kH5N1/loc 1, kH5N1/loc 2, kH5N2/Mex 1, kH5N2/Mex 2,

kH5N2/Pot and kH5N3 were similar to the classical challenge virus with 92, 89, Q "91" M "98", Q "91" M "98", 70, 70, 84, and 91 % respectively. While their similarities to the variant challenge virus with 96, 91, Q "97" M "96", Q "97" M "96", 69, 69, 82 and 93 % respectively.

Table (4): AI vaccine strains similarities to the classical and variant challenge viruses.

Vaccine	Similarity % of classical challenge virus	Similarity % of variant challenge virus
kH5N1/Egy	**92**	**96**
kH5N1/Re-1	**89**	**91**
kH5N1/loc 1*	**Q-91 M-98**	**Q-97 M-96**
kH5N1/loc 2*	**Q-91 M-98**	**Q-97 M-96**
kH5N2/Mex 1	**70**	**69**
kH5N2/Mex 2	**70**	**69**
kH5N2/Pot	**84**	**83**
kH5N3/Viet	**91**	**93**

*****similarities for these types of vaccines were divided into 2 results the 1st results were the similarity of Q-Ag (rg A/Chicken/Egypt/ Q1995D/2010 (H5N1)) while the 2nd results were the similarity of M-Ag (rg A/Duck/Egypt/M2583D/2010 (H5N1)).

II- Invivo evaluation of AI vaccine:

A. Results of protection percents to the classical challenge virus:

The tested vaccines kH5N1/Egy, kH5N1/Re-1, kH5N1/loc 1, kH5N1/loc 2, kH5N2/Mex 1, kH5N2/Mex 2, kH5N2/Pot and kH5N3 yielded protection percents to the classical challenge virus 100, 96, 81.8, 84.8, 90.9, 83, 85 and 89.8% respectively as shown in table (5).

Table (5): Efficacy of different AI vaccine types against the Egyptian classical challenge virus.

Vaccine	Protection % of vaccine batches to the classical challenge virus					Mean protection %
kH5N1/Egy	100	100	100	100	100	**100**
kH5N1/Re-1	100	100	95	95	90	**96**
kH5N1/loc 1	80	80	85	84.2	80	**81.8**
kH5N1/loc 2	90	85	84.2	85	80	**84.8**
kH5N2/Mex 1	100	95	90	89.6	80	**90.9**
kH5N2/Mex 2	90	85	80	80	80	**83**
kH5N2/Pot	90	90	85	80	80	**85**
kH5N3/Viet	95	88.9	90	90	85	**89.8**

B. Results of protection percents to the variant challenge virus:

The tested vaccines kH5N1/Egy, kH5N1/Re-1, kH5N1/loc 1, kH5N1/loc 2, kH5N2/Mex 1, kH5N2/Mex 2, kH5N2/Pot and kH5N3 yielded protection percents to the variant challenge virus 100, 95.9, 81.5, 85, 91 82.9, 85 and 89.9 % respectively as shown in table (6).

Table (6): Efficacy of different AI vaccine types against the Egyptian variant challenge virus.

Vaccine	Protection % of vaccine batches to the variant challenge virus					Mean protection %
kH5N1/Egy	100	100	100	100	100	100
kH5N1/Re-1	100	100	94.7	95	90	95.9
kH5N1/loc 1	80	80	84.2	83.3	80	81.5
kH5N1/loc 2	90	85	85	85	80	85
kH5N2/Mex 1	100	95	90	90	80	91
kH5N2/Mex 2	89.5	85	80	80	80	82.9
kH5N2/Pot	90	90	85	80	80	85
kH5N3/Viet	95	90	89.5	90	85	89.9

III- Relationship between vaccine strain characters and their efficacy for challenge protection:

A. Relationship of results between vaccine strain similarities and the protection percent:

Table (7) showed the result of the similarity percent and the protection percent (as mean for 5 batch/ vaccine type) to the classical and variant challenge viruses. The tested vaccines kH5N1/Egy, kH5N1/loc 2 and kH5N2/Mex 1 were similar to the classical challenge virus with 92, Q"91" M"98", and 70 % respectively. While they were similar to the variant challenge virus with 96, Q"97" M"96"and 69 % respectively. On the other hand, their protection percent to the classical challenge virus were 100, 84.8 and 90.9 % respectively. While their protection percent to the variant challenge virus were 100, 85 and 91 % respectively.

Table (7): The relation between the vaccine strain and protection percent of some inactivated AI vaccines.

Vaccine	No. of batches	Similarity %		Protection %	
		To classical challenge virus	To variant challenge virus	To classical challenge virus	To variant challenge virus
kH5N1/Egy	**5**	**92**	**96**	**100**	**100**
kH5N1/loc 2	**5**	**Q-91 M-98**	**Q-97 M-96**	**84.8**	**85**
kH5N2/Mex 1	**5**	**70**	**69**	**90.9**	**91**

B. Relationship between antigen content and the protection percent:

Data in table (8) described the results of the tested vaccines related to their antigen content and protection percent. The tested vaccines kH5N1/Egy, kH5N2/Mex 1 and kH5N2/Mex 2 and kH5N2/Pot had HA titer 7.6, 7.2, 6.6 and 6.6 log2 in their final products respectively. While they had 1.202×10^5, 5.311×10^4, 2.813×10^4 and 1.595×10^4 copies/140 µl in their final products respectively. Also these vaccines yielded HI titers 8.8, 11.3, 7.8 and 7.4 log2 respectively. On the other hand, these vaccines yielded protection percents to the classical challenge virus 100, 90.9, 83 and 85 % respectively. Also they yielded protection percents to the variant challenge virus 100, 91, 82.9, 85 % respectively.

Table (8): The relation between the antigen content and protection percent of some inactivated AI vaccines.

Vaccine	Viral content in final product			Protection %	
	HA titer (log2)	copies/140 µl by rtRT-PCR	HI titer (log2)	Variant strain	Classical strain
kH5N1/Egy	**7.6**	**1.202×10^5**	**8.8**	**100**	**100**
kH5N2/Mex 1	**7.2**	**5.311×10^4**	**11.3**	**91**	**90.9**
kH5N2/Mex 2	**6.6**	**2.813×10^4**	**7.8**	**82.9**	**83**
kH5N2/Pot	**6.6**	**1.595×10^4**	**7.4**	**85**	**85**
kH5N1/loc 1	**7.4**	**6.505×10^5**	**8.2 "7.4"**	**81.5**	**81.8**

C. Relationship the production method and the protection percent:

From table (9) it was found that, the tested vaccines kH5N1/Egy and kH5N1/Re-1 were produced from reassorteant viral strains but kH5N2/Mex 1and kH5N2/Pot were produced from low pathogenic viral strains. On the other hand, the tested vaccines kH5N1/Egy, kH5N1/Re-1, kH5N2/Mex 1 and kH5N2/Pot yielded protection percent to the classical challenge virus 100, 96, 90.9 and 85 % respectively. Also they yielded protection percent to the variant challenge virus 100, 95.9, 91 and 85 % respectively.

Table (9): The relation between the production method and protection percents of some inactivated AI vaccines.

Vaccine	Type	Protection %	
		Variant	classical
kH5N1/Egy	Reassortant	100	100
kH5N1/Re-1	Reassortant	95.9	96
kH5N2/Mex 1	Low pathogenic	91	90.9
kH5N2/Pot	Low pathogenic	85	85

D. Relationship the vaccine quality and the protection percent:

Data in table (10) showed that, the tested vaccines kH5N1/loc 1, kH5N1/loc 2, kH5N2/Mex 1 and kH5N2/Mex 2 were produced from viral strains rg A/Duck/Egypt/M2583D/2010 (H5N1) and rg A/Chicken/Egypt/Q1995D/2010 (H5N1), rg A/Duck/Egypt/M2583D/2010 (H5N1) and rg A/Chicken/Egypt/Q1995D/2010 (H5N1), A/Chicken/Mexico/232/94 /CPA H5N2 LPAI and A/Chicken/Mexico/232/94 /CPA H5N2 LPAI respectively. On the other hand, these vaccines yielded protection percents to the classical challenge virus 81.8, 84.8, 90.9 and 83 % respectively. Also they yielded protection percents to the variant challenge virus 81.3, 84.8, 91 and 82.9 % respectively.

Table (10): The relation between the vaccine quality and the protection percents of some inactivated AI vaccines.

Vaccine	AI strain	protection %	
		Variant	Classical
kH5N1/loc 1	rg A/Duck/Egypt/M2583D/2010 (H5N1) rg A/Chicken/Egypt/ Q1995D/2010 (H5N1)	81.5	81.8
kH5N1/loc 2	rg A/Duck/Egypt/M2583D/2010 (H5N1) rg A/Chicken/Egypt/ Q1995D/2010 (H5N1)	85	84.8
kH5N2/Mex 1	A/Chicken/Mexico/232/94 /CPA H5N2 LPAI	91	90.9
kH5N2/Mex 2	A/Chicken/Mexico/232/94 /CPA H5N2 LPAI	82.9	83

DISCUSSION

Vaccine evaluation is considered one of the critical points helping veterinarians in their decisions to select the most suitable vaccine type used in the vaccination programs in the field. The aim of AI vaccination is the production of an immune response that is protective against the disease and prevention of infection. The assessment of that goal is important for vaccine licensing (Swayne, 2006). A variety of vaccines are used to control the disease in chickens as inactivated whole AI virus oil emulsion vaccines (Lin *et al.,* 2006) and vectored vaccines either fowl Pox (Paoletti, 1990), Herpes (Gardin *et al.,* 2016) and ND virus (Swayne *et al.,* 2003). In this study, 8 imported and locally produced inactivated AI vaccines were comparatively studied. They were the most prominent AI inactivated vaccines used in the Egyptian field. These vaccines were evaluated through *invivo* and *invitro* tests. The aim from these comparative results of the tested vaccines was to detect the correlation between the vaccine formulation (as vaccine strain type and content) and their ability to induce good protection percent in the vaccinated birds. Also, to clear the role of *invitro* methods in the vaccine evaluation in comparison with the *invivo* pathway (vaccine-challenge method). The *invitro* tests usually have the advantage of being time and cost saving and simpler than the ordinary *invivo* tests due to absence of usage of animals and observation of complex clinical signs Maas *et al.,* (2000). But, it is necessary to initially demonstrate that if the vaccine strain is protective against the specific field strain or not before applying these indirect assessment of vaccine efficacy (Swayne, 2006). So, indirect assessment can be a viable option in some situations to assess protection as when determining the consistency of vaccine batches as a mean to insure a minimal protective level (Swayne, 2006). Also, the indirect methods is very important way to directly detect the vaccine strain identity and antigen content in the final inactivated AI vaccines without using vaccination system.

The vaccine strain included in the vaccines under study were identified *invivo* (indirect method) by HI test through testing of the collected sera from vaccinated chickens against reference antigens for AI (H5), AI (H9), ND and EDS. Also they were identified *invitro* directly by rRT-PCR technique using specific sets of primers against AI (H5, H9, N1, N2 and N3) and NDV (F gene) as described by Wise *et al.,* (2004); LÖndt *et al.,* (2008) and Ben Shabat *et al.,* (2010).The obtained results (by both the *invivo* and *invitro* methods) approved that the vaccine strains in the tested vaccines were H5 positive and negative for H9, NDV and EDS. But, it was observed that the *invitro* method has the advantages of being direct, rapid, low cost and did not

need host inoculation like *invivo* method. The previous results agreed with Tang *et al.,* (2005) who said that HI test used *invivo* method is the most convenient, rapid and economical serological test for detection of AI virus. But in recent years, the application of molecular methods for detection of viral nucleic acids has become an important, high speed and low cost tool for the detection of AI virus (Poddar 2002 and Iqbal et al., 2013).

Laboratory models can be useful as a direct parameter for vaccine evaluation when variables such as virus strain and antigen content are standardized (Swayne *et al.,* 1999). In addition, a variety of indirect measures can be used as a pointer for protection when compared to the *invivo* protection data. One of these measures that assays used to quantify the amount of immunologically protective protein in the vaccine (Maas *et al.,* 2000). Quantification of antigen in inactivated vaccines has been accomplished by heamagglutinating titer (Swayne *et al.,* 1999), rRT-PCR assay (Swayne *et al.,* 2006) and infectious titer prior to inactivation (Swayne *et al.,* 1999).

In this study the viral content were quantified *invitro* from the tested final product vaccines through measuring the HA titer of the eluted antigen phase by isopropyl myrestate and nucleic acid copies by rRT-PCR using trizol LS. It was found that the HA titers was ranged from 6 to 7.6 log2 for all the inactivated AI vaccines (table. 3), while the rRT-PCR titer of them ranged from 1.484×10^4 to 6.505×10^5 copies/140 µl.

Also, the viral content of all types of the tested inactivated vaccines was estimated by indirect way through detection of HI titer of the sera collected from the vaccinated chickens (*invivo* method). The HI results at the 4^{th} WPV were 8.8, 8, 8.2 "7.4", 7.8 "7.1", 11.3, 7.8, 7.4 and 8.5 log 2 for kH5N1/Egy, kH5N1/Re-1, kH5N1/loc 1, kH5N1/loc 2, kH5N2/Mex 1, kH5N2/Mex 2, kH5N2/Pot and kH5N3/Viet respectively. It was noticed that there was a correlation between the high viral content and the high HI level produced by the same vaccines.

This is obvious in case of inactivated kH5N1/Egy which had 9 log2 HA titer and 8.5 EID_{50}/ml before inactivation and 7.6 log2 HA titer in final product, it would produce high HI titer in the sera of vaccinated chicken (8.8 log2) as shown in table (3). But other vaccines like kH5N2/Mex 1 which had HA titer lower than the kH5N1/Egy (8 log2 before inactivation and 7.2 log2 in the final product), would produce 11.3 log2 HI titer. This is parallel to Thornton, (1988) who said that sufficient HA antigen must be present in the inactivated vaccines to produce a serologically measurable protective response and there should be minimal batch-to-batch variation in antigen content.

Also, Garcia *et al.,* (1998) said that the vaccine should be formulated with sufficient HA antigen to produce a consistent protective response. Their results suggested a single immunization dose of 4 µg or greater of HA protein induced immune response that was the best for reducing replication of challenge virus. But they found that all the tested inactivated vaccines of the same vaccine strains provided similar good protection from mortality while individual vaccines varied in the ability to reduce shedding with the same degree and to induce the same serological response.

Also, the *invivo* method for evaluation of the inactivated AI vaccines efficacies was the direct assessment of protection % of them using the LPAI or HPAI virus challenge models (Xie and Stone, 1990). Challenge test is considered the standard method to assess the protection through detecting the percent of prevention of respiratory and general clinical signs (morbidity) and deaths (mortality) against HPAI virus (Brugh *et al.,* 1979, Wood *et al.,* 1985, Stone, 1987 and Stone, 1988).

In this study, it was found that all chickens vaccinated with the tested AI vaccines were challenged by 2 kinds of HPAI viruses, one belong to the classical virus group (A/Ch/Egypt/Qal-3/2016 (H5N1)) and the other related to the variant group (A/Ch/Egypt/1709-6/2008 (H5N1)).

The mean protection percent of all the inactivated AI vaccines was ranged from 81.8% and 81.5% (for kH5N1/loc 1) to 100% (for kH5N1/Egy) against both classical and variant challenge viruses respectively (tables 5 and 6). However, it was found that some vaccines like kH5N1/Egy that had 7.6 log2 HA titer, 1.202 x 10^5 rRT-PCR copies and 8.8 log2 HI titer induce 100% protection against both classical and variant challenge viruses (table. 8). While, another vaccine as kH5N2/Mex 1 which have lower viral content (7.2 log2 HA titer and 5.311 x 10^4 rRT-PCR copies) but high HI titer (11.3 log2) induced lower protection percent than kH5N1/Egy (90.9% and 91% for classical and variant challenge viruses respectively) as shown in table (8). Other vaccine like kH5N1/loc 1 had high antigen content equal 7.4 log2 HA titer and 6.505 x 10^5 rRT-PCR copies could protect only 81.8% and 81.5% of vaccinated chickens against both classical and variant challenge viruses respectively.

This data was matched with (Swayne *et al.,* 1997 and Swayne *et al.,* 1999) that said that the high content of HA in inactivated vaccines or high titer of live vaccine strains before inactivation provides the best protection against AI replication in the respiratory and digestive tracts. While the lower content vaccines may protect from morbidity but not reduce replication and shedding.

On the other hand, Vogel, (2000) observed that there were many factors as adjuvants type are important in activation and directing the innate and adaptive immune responses to the rather poorly immunogenic inactivated vaccine antigens.

The present work also describes the effect of vaccine strain similarities to the field HPAI viruses on the protection percent induced by the tested inactivated AI vaccines.

It was observed from table (4) that the similarity between the vaccine strains of inactivated reassortant vaccines as kH5N1/Egy, kH5N1/Re-1, kH5N1/loc 1, kH5N1/loc 2, and kH5N3/Viet was ranged 92, 89, “91, 98”, “91, 98” and 91 % to the classical challenge virus respectively. While, the identity of the same vaccine strains with the variant challenge virus was 96, 91, “97, 96”, “97, 96” and 93 % respectively.

These results approved that the similarity between the vaccine strains in the inactivated AI vaccines and the circulating challenge viruses is very important in induction of good protection percent in the vaccinated birds. It was clear in case of kH5N1/Egy vaccine which induce 100% protection. Against both classical and variant HPAI challenge virus (table 7).

But, although the similarity between the vaccine strains in the kH5N1/loc 1 to the challenge viruses was ranged from 91-98%, the vaccine could protect only 85% of the vaccinated birds against that HPAI viruses.

Also, the vaccine strain of kH5N2/Mex 1 was identical to the classical and variant challenge viruses with 70 to 69 %. But it could protect the birds against them with a protection percent reach to 91%.

These results were agreed with (Swayne *et al.,* 2000) who found that the closer the HA gene sequence similarity between the vaccine and field viruses, the greater was the protection and reduction in challenge virus replication in the respiratory tract.

On the other hand, Swayne, and Akey, (2005) showed that the inactivated oil adjuvant vaccines have been far less affected by drift in the field viruses. this means that the H5 vaccine strains have provided broad cross-protection from mortality against H5 HPAI field viruses that collected over 38 years and differing by as much as 12% in amino acid sequences at HA1compared to the challenge HPAI virus (Swayne *et al.,* 1997, Swayne *et al.,* 2000, Swayne *et al.,* 2001). So, that data explain the issue of a good quality vaccine can protect the chickens against many different challenge viruses with the same level (e.g. kH5N1/Egy produce 100% protection percent against classical and variant HPAI) as mentioned by Kim *et al.,* (2008).

This broad protection is not absolute within all subtypes, e.g. the inactivated H5N2 vaccine used in Mexico from 1995 till 2007 contains vaccine strain LPAI Mexican H5N2/94 and provided protection in chickens against HPAI field virus isolated at 1995 (Garcia *et al.,* 1998 and Lee *et al.,* 2004). But the same vaccine strain was not protective against two later lineages of H5N2 LPAI isolated in1998 and 2003 (Lee *et al.,* 2004). So it is unclear whether the drift in the field viruses resulted from immunity following infections in nonvaccinated poultry or improper vaccination.

This means that, there are other parameters which increase vaccine efficacy as good manufacturing procedures as shown with the kH5N2/Mex 1 and kH5N2/Mex 2 or kH5N1/loc 1 and kH5N1/loc 2 which have the same vaccine strains but produce a variable protection% (table). Also, the proper adjuvant system, biosafety and biosecurity facilities and the rout of immunization play an important role in vaccine efficacy (OIE, 2017).

CONCLUSION

The results illustrated that sufficient HA antigen and similar HA sequences with the field HPAI isolates are important to produce a consistent protective responses, but the detection of them is not enough to assess the vaccine efficacy.

Also, the indirect assessment of protection not considered an alternative methods to the direct assessment of efficacy but may be used as complementary steps for vaccine evaluation in a final product case. Especially in direct detection of vaccine strain identification and to somewhat quantification in the oil emulsion inactivated vaccine.

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RECEIVED: May 2018; **ACCEPTED**: June 2018; **Published:** July 2018

REFERENCES

Abdelwhab, E.M.; Arafa, A.S.; Stech, J.; Grund, C.; Stech, O.; Graeber-Gerberding, M.; Beer, M.; Hassan, M.K.; Aly, M.M.; Harder, T.C. and Hafez, H.M. 2012. Diversifying evolution of highly pathogenic H5N1 avian influenza virus in Egypt from 2006 to2011. Virus Genes (2012), 45:14–23.

Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W. and Lipmanl, D.J. 1990. Basic local alignment search tool. Mol. Biol. Evol., 215: 403-410.

Arafa, A.M.; El-Masry, I.; Kholosy, S.; Hassan, M.K.; Dauphin, G.; Lubroth, J. and Makonnen, Y.J. 2016. Phylodynamics of avian influenza clade 2.2.1 H5N1 viruses in Egypt**.**Virology Journal (2016) 13:49.DOI 10.1186/s12985-016-0477-7

Arafa, A.S.; Naguib, M.M.; Luttermann, C.; Selim, A.A.; Kilany, W.H.; Hagag, N.. 2015. Emergence of a novel cluster of influenza A (H5N1) virus clade 2.2.1.2 with putative human health impact in Egypt, 2014/15. Euro Surveill. (2015), 20(13):2–8.

Awad, E.T.; Gouda, E.; El-Husseiny, M.H.; Aly, M.M.; Pushko, P.; Tretyakova, I. and Arafa, A.M. 2015. Biochemical and immunogenicity studies on Hemagglutinin protein rescued from H5N1 avian influenza virus like particles. Journal of American Science 2015;11(4). http://www.jofamericanscience.org

Ben Shabat, M.; Meir, R.; Haddas, R.; Lapin, E.; Shkoda, I. Raibstein, I.; Perk, S. and Davidson, I. 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J Virol Methods.; 168(1-2): 72-7.

Brugh, M.; Beard, C.W. and Stone, H.D. 1979. Immunization of chickens and turkeys against avian influenza with monovalent and polyvalent oil emulsion vaccines. American Journal of Veterinary Research 40:165-169.

Das, A.; Spackman, E.; Senne, D.; Pedersen, J. and Suarez, D.L. 2006. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized agents. J. Clin. Microbial. 44:3065-3073.

El-Zoghby, E.F.; Arafa, A.M.; Kilany, W.H.; Aly, M.M.; Abdelwhab, E.M. and Hafez, H.M. 2012. Isolation of avian influenza H5N1 virus from vaccinated commercial layer flock in Egypt. Virology Journal .2012, 9:294. http://www.virologyj.com/content/9/1/294

Garcia, A.; Johnson, H.; Srivastava, K.; Jayawardene, A.; Wehr, R. and Webster, G. 1998. Efficacy of inactivated H5N2 influenza vaccines against lethal A/Chicken/Queretaro/19/95 infection. Avian Dis., 42:248–56.

Gardin, Y.; Palya, V.; Dorsey, K.M.; El-Attrache, J.; Bonfante, F.; de Wit, S.; Kapczynski, D.; Kilany, W.H.; Rauw, F.; Steensels, M. and Soejoedono, R.D. 2016. Experimental and field results regarding immunity induced by a recombinant turkey herpesvirus H5 vector vaccine against H5N1 and other H5 highly pathogenic avian influenza virus challenges. Avian Diseases, 60(1s): 232-237.

Hafez, M.H.; Arafa, A.; Abdelwhab E.M.; Selim, A.; Khoulosy, S.G.; Hassan, M.K. and Aly, M.M. 2010. Avian influenza H5N1 virus infections in vaccinated commercial and backyard poultry in Egypt. Poult Sci (2010), 89:1609–1613.

Hussein, H.A.; Emara, M.M. and Rohaim, M.A. 2015. Genetic diversity of avian influenza H5N1 subclade 2.2.1/c in commercial poultry in Egypt during 2013. Global Veterinaria 2015; 14(5): 662-669.

Ibrahim, M.; Eladl, A.F.; Sultan, H.A.; Abdel Satar Arafa, A.M.; Abdel Razik, A.G.; Abd El Rahman, S.; Abou El-Azm, K.L.; Saif, Y.M.; and Lee, C.W. 2013. Antigenic analysis of H5N1 highly pathogenic avian influenza viruses circulating in Egypt (2006–2012). Veterinary Microbiology (2013) Dec 27; 167(3-4):651-661.

Iqbal, M.; Yaqub, T.; Mukhtar, N.; Shabbir, M.Z. and McCauley, J.W. 2013. Infectivity and transmissibility of H9N2 avian influenza virus in chickens and wild terrestrial birds. Vet. Res., 44:100.

Kapczynski, D.R.; Esaki, M.; Dorsey, K.M.; Jiang, H.; Jackwood, M.; Moraes, M. and Yannick Gardin, Y. 2015. Vaccine protection of chickens against antigenically diverse H5 highly pathogenic avian influenza isolates with a live HVT vector vaccine expressing the influenza hemagglutinin gene derived from a clade 2.2 avian influenza virus. Vaccine 33 (2015): 1197–1205.

Kayali, G.; Kandeil, A.; El-Shesheny, R.; Kayed, A. S.; Maatouq, A.M.; Cai, Z.; McKenzie, P.P.; Webby, R.J.; El Refaey, S.; Kandeel, A.; Ali, M.A. 2016. Avian Influenza A (H5N1) Virus in Egypt. www.cdc.gov/eid. Emerging Infectious Diseases. Vol. 22, No. 3, March 2016; 379-388.

Kim, J.K.; Seiler, P.; Forrest, H.L.; Khalenkov, A.M.; Franks, J.; Kumar, M.; Karesh, W.B.; Gilbert, M.; Sodnomdarjaa, R.; Douangngeun, B.; Govorkova, E.A. and Webster, R.G. 2008. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. J. Virol. Vol., 82: 11374-11382.

Lee, C.W.; Senne, D.A. and Suarez, D.L. 2004. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J. Virol., 78:8372-8381.

Lin, J.; Zhang, J.; Dong, X.; Fang, H.; Chen, J.; Su, N.; Gao, Q.; Zhang, Z.; Liu, Y.; Wang, Z.; Yang, M.; Sun, R.; Li, C.; Lin, S.; Ji, M.; Wang, X.; Wood, J.; Feng, Z.; Wang, Y.;

and Yin, W. 2006. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomized controlled trial. Lancet, 368: 991-997.

Lòndt, B.Z.; Nunez, N.; Banks, J.; Nili, H.; Johnson, L.K. and Alexander, D.J. 2008. Pathogenesis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (Anas platyrhynchos) infected experimentally. Avian Pathology (December 2008) 37(6), 619_627.

Maas, P.A.; de Winter, M.P.M.; Venema, S.; Oei, H.L. and Claassen, I.J.T M. 2000. Antigen quantification as in vitro alternative for potency testing of inactivated viral poultry vaccines, Veterinary Quarterly, 22:4, 223-227.

Maas, R.A.; Komen, M. van Diepen, M.; Oei, H.L. and Claassen, I.J. 2003. Correlation of haemagglutinin-neuraminidase and fusion protein content with protective antibody response after immunisation with inactivated Newcastle disease vaccines. Vaccine. 2003 Jul 4; 21(23): 3137-42.

OIE [Office International des Epizoties]. 2017. Chapter 2.3.4. In: Manual of diagnostic tests and vaccines for terrestrial animals. Version adopted by the World assembly of Delegates of the OIE in May 2015. http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza.

Paoletti, E. 1990. Poxvirus recombinant vaccines. Ann. N.Y. Acad. Sci., 590: 309–325.

Peyre, M.; Samaha, H.; Makonnen, Y.J.; Saad, A.; Abd-Elnabi, A.; Galal, S.; Ettel, T.; Dauphin, G.; Lubroth, J.; Roger, F.; Domenech, J.; 2009. Avian influenza vaccination in Egypt: limitations of the current strategy. J. Mol. Genet. Med. (2009) 3, 198–204.

Poddar, S.K. 2002. Influenza virus types and subtypes detection by single step single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis. J. Virol. Meth. 99(1-2): 63-70.

Stone, H.D. 1987. Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. Avian Diseases 31: 483-490.

Stone, H.D. 1988. Optimization of hydrophile-lipophile balance for improved efficacy of Newcastle disease and avian influenza oil-emulsion vaccines. Avian Dis., 32:68-73.

Swayne D.E. 2006. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1high pathogenicity avian influenza. Annals of the New York Academy of Sciences 1081:174-181.

Swayne, D.E. and Akey, B. 2005. Avian influenza control strategies in the United States of America. In: Schrijver, R. S. and Koch, G. (ed.). Avian influenza. Prevention and control. Springer: Dordrechet, pp. 113-130.

Swayne, D.E. and Halvorson, D.A. 2003. Influenza. In: Saif, Y. M.; Barnes, H. J.; Fadly, A. M.; Glisson, J. R.; McDougald, L. R. and Swayne, D. E. (eds.). Diseases of poultry, 11th ed. Iowa State University Press: Ames, IA, pp. 135-160.

Swayne, D.E.; Beck, J.R. and Mickle, T.R. 1997.
Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-Origin H5N2 avian influenza virus. Avian Diseases 41(4): 910-922.

Swayne D.E.; Beck, J.R.; Garcia, M. and Stone, H.D. 1999. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian pathology 28: 245-255.

Swayne, D.E.; Beck, J.R.; Perdue, M.L. and Beard, C.W. 2001. Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. Avian Dis., 45(2): 355 – 365.

Swayne, D.E.; Garcia, M.; Beck, J.R.; Kinney, N. and Suarz, D.L. 2000. Protection against diverse highly pathogenic avian influenza viruses in chickens immunized with a

recombinant fowl pox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine 18(11-12): 1088-1095.

Swayne, D.E.; Lee, C-W. and Specman, E. 2006. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian pathology. 2006. 35 (2). 141-146.

Swayne, D.E.; Suarez, D.L.; Schultz-Cherry, A.S.; Tumpey, T.M.; King, D. J.; Nakaya, A. T.; Palese, B. P. and Garcia-Sastre, A. B. 2003. Recombinant Paramyxovirus Type 1-Avian Influenza-H7 Virus as a Vaccine for Protection of Chickens against Influenza and Newcastle Disease. Avian Diseases, 47:1047–1050.

Tang, Y.; Lee, C.W.; Zhang, Y.; Senn, D.A.; Dearth, R. and Byram, B. (2005). Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis., 49: 207-213.

Thornton, D.H. 1988. Quality control of vaccines. In: Alexander, D.J. (ed.). Developments in Veterinary Virology: Newcastle Disease. Kluwer Academic Publishers: Dordecht, the Netherlands, pp. 347-365.

Vogel, F.R. 2000. Improving vaccine performance with adjuvants. Clinical Infectious Diseases. 30(Suppl.3):S266-S270.

WHO/OIE/FAO H5N1 Evolution Working Group, 2012. Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza Other Respir. Viruses 6, 1–5.

WHO/ OIE/ FAO H5N1 Evolution Working Group, 2014. Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses. Influenza Other Respir Viruses. (2014), 8(3):384–8.

Wise, M.G.; Suarez, D.L.; Seal, B.S.; Pedersen, J.C.; Senne, D.A.; King, D.J.; Kapczynski, D.R. and Spackman, E. 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J. Clin. Microbiol.42: 329–338.

Wood, J.M.; Kawaoka, Y.; Newberry, L.A.; Bordwell, E. and Webster, R.G. 1985. Standardization of inactivated H5N2 influenza vaccine and efficacy against lethal A/chicken/Pennsylvania/1370/83 infection. Avian Dis., 29:867-872.

Xie, Z.X. and Stone, H.D. 1990. Immune response to oil emulsion vaccines with a single or mixed antigens of Newcastle, avian influenza and infectious bronchitis. Avian diseases, 34:154-162.

Cite this article as:
Marden ***et al.,*** **(2018)**: Comparative Studies on Evaluation of Different Avian Influenza Inactivated Vaccines under Local Conditions in Egypt., *J. of Virol. Sci.,* Vol. 4: 57-71, 2018.