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Efficacy of inactivated avian influenza H5N1 vaccine in SPF chicken against Egyptian isolates of avian influenza viruses H5N2 and H5N8

El-Bagoury, G. F1, El-Nahas E. M.,1 EL-Safty,M.M. 2 and Faten M. A,2

[1]*Departments of virology, Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt.*

[2]*CLEVB, SPF department, Abbassia, Cairo, Egypt.*

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ABSTRACT

In the present study available inactivated avian influenza vaccine H5N1 was evaluated for its efficacy against challenging with the recently isolated HPAI H5N2 (A/chicken/EG/16194V/2016) and H5N8 (HPAI clade 2.3.4.4 (A/green-wingedteal/ Egypt/871/2016(H5N8) (871/H5N8). our study had been achieved using SPF chickens which were divided into four groups .They were vaccinated at one , five , ten and twelve day old, then serologically monitoring on a weekly basis post vaccination (PV) for the 1st month, and follow immunity every month till the 6th month using Haemagglutination Inhibition (HI) test against H5N1 HPAIclade2.2.1.2 (A/chicken/Egypt/D10552B/2015 (H5N1) (D10552BH5N1), H5N2(A/chicken/EG/16194V/2016) and H5N8 HPAIclade2.3.4.4 (A/green winged teal/Egypt/871/2016 (H5N8) (871/H5N8), then challenge test were conducted at 2nd ,3rd and 4th weeks PV using the recently isolated H5N2 and H5N8 field strains challenge viruses , tracheal and cloacal swabs were collected for detection of virus shedding . Our results indicate that the vaccine provides protection against different subtypes of viruses and decreases virus shedding from the challenged chicken when chickens vaccinated at twelve day old challenged after four weeks post vaccination.

1. INTRODUCTION

Avian Influenza virus (AIV) is an important poultry pathogen and a massive menace to the poultry industry. Highly Pathogenic Avian Influenza (HPAI) H5N1 affects the poultry industry in many countries since 1990s. After 2004, H5N1 has spread from Asia to all over the world leading to killing or culling of millions of domestic birds (Li et al., 2004).

Continuous circulation of the AIV in both vaccinated and non-vaccinated commercial and backyard poultry was reported in Egypt, although vaccination strategy of poultry flocks in order to combat H5N1 AI V (Hafez et al., 2010).

One of the main causes of vaccination failure as a control measure is the variation in AIV antigenicity which develops gradually by point mutation (antigenic drift) (Cattoli et al., 2011) or drastically by genetic re-assortment (antigenic shift) (Bouvier and Palese, 2008).

Antigenic analysis of H5N1 strains in Egypt demonstrated considerable variations (Balish et al., 2010) with circulation of stable lineages of H5N1 viruses since late 2007.

Vaccination against H5N1 has become one of the most important control measures for HPAI in poultry industry since 2006 (OIE, 2010)

The aim of this work is to evaluate the efficacy of of the inactivated H5N1 AI vaccine in chicken against challenging with the recently isolated HPAI H5N2 (A/chicken/EG/16194V/2016) and H5N8 (A/green-winged teal/ Egypt/871/2016 (H5N8) (871/H5N8) field isolate strains.

2. MATERIAL AND METHODS

2.1. Experimental chicks

A total number of 480 Specific Pathogen Free (SPF) one day old chicks were provided by Qoum Oshim SPF farm, Fayum, Egypt. During the experiment period, the chicks were housed in BSL3 chicken isolators. The chicks were housed in good hygienic conditions and were ventilated under negative pressure with HEPA- filtered air. Continuous lightening; feed and water should be supplied. Daily monitoring for chicken groups were done all over the experiment.

2.2. Vaccine and Viruses

Avian influenza H5N1 virus vaccine: Inactivated imported commercial bivalent vaccine (Each Dose 0.5 ml contains H5N1 classic Strain 2.2.1.2, and H5N1 Variant Strain 2.2.1.1.batch number: 1901230101).

Viruses: (1) HPAI H5N1 (A/Chicken/Egypt/ D10552B/2015 (H5N1)) (D10552B (H5N1)) Egyptian field strain with titer was $10^{8.5}$ EID50. (2) HPAI H5N2 (A/chicken/EG/ 16194V/2016) Egyptian field strain with titer was $10^{8.0}$ EID50.

(3) HPAI H5N8 (A/green-winged teal Egypt/871/2016 (H5N8) (871/H5N8) Egyptian field strain with titer was $10^{9.0}$ EID50.

All viruses used in HI test were submitted by Reference lab for veterinary quality control on poultry production (RLQp) to Central lab for evaluation of veterinary

Correspondence to: fatenm98@yahoo.com

biologics. The host of adaption was specific pathogen free embryonated chicken eggs and chickens. Only H5N2V and H5N8V used as challenge viruses. They were used as challenge virus with a titer 10^6 EID50 and inoculated 0.1ml /bird intranasal. (Spackman and Killian, 2014).

Four hundred and eighty SPF chicks were divided into four groups (A, B, C and D). They were vaccinated with H5N1 vaccine at (1, 5, 10 and 12 day old, respectively) . Each group was divided into six subgroups in addition to control subgroup. Experimental design is described in table (1). Vaccination was based on manufacturer recommendation dose. The chickens were injected with 0.5 ml S/C with the inactivated H5N1 vaccine. Daily observation for all chicken groups and record any clinical signs and mortalities.

Table (1) The experimental design

Sub group	n	Day of vaccination	Challenge group	n	Day of challenge	Challenge strain
A	120	1	A1+control	10+10	2 wpv	H5N2
			A2+control	10+10		H5N8
			A3+control	10+10	3 wpv	H5N2
			A4+control	10+10		H5N8
			A5+control	10+10	4 wpv	H5N2
			A6+control	10+10		H5N8
B	120	5	B1+control	10+10	2 wpv	H5N2
			B2+control	10+10		H5N8
			B3+control	10+10	3 wpv	H5N2
			B4+control	10+10		H5N8
			B5+control	10+10	4 wpv	H5N2
			B6+control	10+10		H5N8
C	120	10	C1+control	10+10	2 wpv	H5N2
			C2+control	10+10		H5N8
			C3+control	10+10	3 wpv	H5N2
			C4+control	10+10		H5N8
			C5+control	10+10	4 wpv	H5N2
			C6+control	10+10		H5N8
D	120	12	D1+control	10+10	2 wpv	H5N2
			D2+control	10+10		H5N8
			D3+control	10+10	3 wpv	H5N2
			D4+control	10+10		H5N8
			D5+control	10+10	4 wpv	H5N2
			D6+control	10+10		H5N8

2.3. Serological monitoring of antibodies

Blood samples were collected from jugular vein, and then kept at 37 ºC for one hour after that blood samples were refrigerated at 4 ºC overnight. Sera were separated by centrifugation at 3000 rpm for 10 minutes then stored at -20 ºC till being used. Inactivation of sera was applied at 56 ºC for 30 minutes before testing. Sera were sampled every week after vaccination for four weeks for the 1st month then every month till the 6th month after vaccination. HI test was done on serum samples using homologous H5N1 and heterologus H5N2 and H5N8 antigens following OIE, (2015).

2.4. Challenge of vaccinated chickens and isolation of shed virus

Each group were challenged intranasally (100 ul contain 10^6 EID50/chicken) with H5N2 and H5N8 antigens as described before in experimental design. All over the experiment the chickens were in BSL3 chicken isolators with daily observation for 10 days post challenge to record the clinical sings, mortalities and virus shedding titer detection. Tracheal and cloacal swabs were taken at 3rd, 5th, 7th and 10th day post challenge. Results of shedding were calculated according to Spearman-Karber method (1961). For virus re-isolation in ECE, The oropharyngeal and cloacal swabs were detected in embryonated chicken egg. Both (oropharyngeal and cloacal swabs) were stored in isotonic phosphate buffered saline (pH 7.0) with antibiotics (Penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml)) following OIE, (2015).

For virus inoculation in ECE, these suspensions filtered through 0.22µm filter. Five 9- 11 day-old SPF ECE were inoculated and candled daily for embryo viability for 7 days (Beard et al., 1989). The dead eggs were discarded within 24 hours. Allantoic fluid from ECE and tested for the presence of AI H5 virus by rapid slide HI test (Anon et al., 1971).

2.5. Virus shedding

Virus shedding titers were detected by both challenge virus re-isolation in ECE for tracheal and cloacal swabs on the 3rd, 5th, 7th, and 10th days post challenge.

3. RESULTS

3.1. Immunogenicity of H5N1 vaccine in SPF chicken's groups

Immune response of all vaccinated chicken groups was increased significantly to reach the highest titer by the fourth week post vaccination then declined till the 6th month post vaccination.

3.2. Immune response of chicken vaccinated with inactivated H5N1 vaccine using HI test against H5N1 virus:

Chickens vaccinated at twelve day old (group D) showed higher mean antibody titers at the 4th WPV recording 7.9 log 2 against H5N1 Ag (antigen; then this titer decline gradually to reach 2 log 2 at the 6th month post vaccination. Chickens vaccinated at ten day old (group C) were 7 log 2 as a higher titer at the 4th WPV then decreased gradually until reach 1.6 log2 at the 6th month post vaccination.

Serum antibody titer of chicken in group A and group B vaccinated at one and five-day old recorded 6.2log 2, and 6.9log 2; respectively, then these titers decline gradually from the 4th week post vaccination to reach 1.0log 2, and 1.6 log2 at the 6th month post vaccination, respectively (fig. 1)

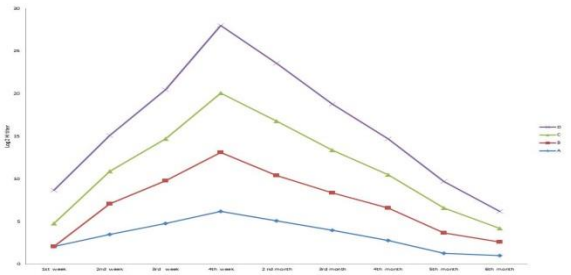

Fig.1. HI titer of chicken gps. vaccinated with H5N1 vaccine at different ages using H5N1 Ag

3.3. Immune response of chicken vaccinated with inactivated H5N1 vaccine using HI test against H5N2 Antigens:

Chickens which were vaccinated at twelve day old (group D) showed increase in antibody titers at the 4th WPV recording 6.0 log 2 against H5N2 Ag., then decline gradually to reach 1.0 log 2 at the 6th month. Chickens vaccinated at ten day old (group C) recording 6.4 log 2 as a higher titer at the 4th WPV then decreased gradually until reach 1.0 log2 at the 6th month. Serum antibody titers of chicken in (group A and group B) which were vaccinated at one and five day old recorded 5.2log 2, and 5.6 log 2 titers, respectively 4th WPV then decline gradually to reach 0log 2,0 log2 at the 6th month (fig.2).

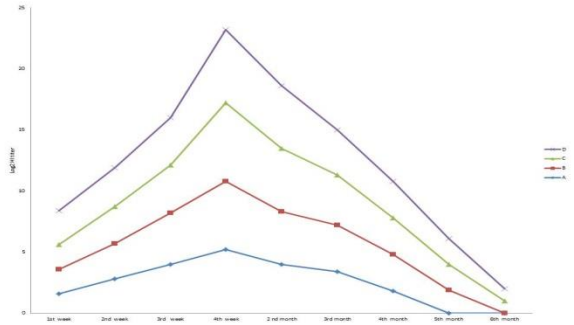

Fig.2.HI titer of chicken gps.vaccinated at different ages using H5 N2Ag

3.4. Immune response of chicken vaccinated with inactivated H5N1 vaccine using HI test against H5N8:

Chickens vaccinated with H5N1 vaccine at twelve day old (group D) showed increase in antibody titers at the 4th WPV recording 6.8 log^2 against H5N8 Ag., then this antibody titer declined gradually to reach 1.8log 2 at the 6th month. Chickens vaccinated at ten day old (group C) were 6.7log_2 as a higher antibody titer at the 4th WPV, then decreased gradually until reach 1.4log2 at the 6th month. Serum antibody titer of chicken in (group A and group B) which were vaccinated at one and five day old recorded 5.8 log 2, and 5.2log 2, respectively at the 4th WPV, then these titers declined gradually to reach 0log 2,0 log2 , respectively at the 6th month (fig.3).

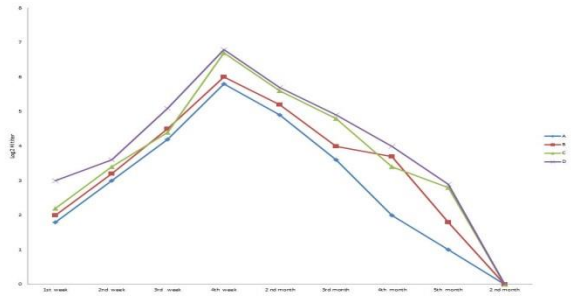

Fig.3. HI titer of chicken gps. vaccinated at different ages using H5N8 Ag

3.5. Challenge of chicken groups:

Chickens were challenged using Egyptian HPAIV H5N2 and H5N8 viruses at 2nd 3rd and 4th WPV,. About 10 chickens for each group were infected. Only live chickens were monitored to detect virus shedding by titration of Log_{10} EID_{50}/mL for each collected sample. All the experiment was applied at CLEVB using reference Egyptian strains. The chickens (D5 gp.) vaccinated with H5N1 vaccine showed 80 % protection against (H5N2) virus and 60 % (D6 gp.) protection against (H5N8) as a higher protection result. Results are recorded in table (2) and table (3).

Table (2):- Protection results of chicken Groups challenged with H5N2

Group	Protection%	control
A1	10%	0%
A3	20%	0%
A5	45%	0%
B1	10%	0%
B3	30%	0%
B5	50%	0%
C1	20%	0%
C3	50%	0%
C5	60%	0%
D1	30%	0%
D3	60%	0%
D5	80%	0%

A: chicken Groups vaccinated at 1day old , B: chicken Groups vaccinated at 5day old, C: chicken Groups vaccinated at 10 day old , D: chicken Groups vaccinated at 12 day old, A1, B1, C1 and D1: challenged after 2nd WPV, A3, B3, C3andD3: challenged after 3rd WPV, A5, B5, C5 and D5: challenged after 4th WPV

Table (3): Protection results of chicken Groups challenged with H5N8

Group	Protection%	control
A2	0%	0%
A4	10%	0%
A6	30%	0%
B2	0%	0%
B4	20%	0%
B6	40%	0%
C2	20%	0%
C4	40%	0%
C6	50%	0%
D2	20%	0%
D4	50%	0%
D6	60%	0%

A2, B2, C2 and D2: challenged after 2nd WPV, A4, B4, C4andD4: challenged after 3rd WPV,A6, B6, C6 and D6: challenged after 4th WPV

By the 3rd day post infection, all control chickens challenged by (H5N2) virus with typical symptoms of highly pathogenic infection including cyanotic combs and wattles, edema of the head, and shank of leg hemorrhage, while the control chickens challenged with (H5N8) virus died by 7th day post infection

3.6. Clinical signs:

The characteristic clinical signs for HPAI observed 3 days post challenge with mortalities occurred in different challenged groups against H5N2 Ag. Sick birds displayed cyanosis of comb and wattle, ecchymosis on the shanks and feet, facial edema, greenish diarrhea and nervous signs including torticollis and tremors. We found that the best protection result was in (Group D). Mortalities are described in tables (4 and 5).

3.7. Virus shedding:

A higher viral shedding was detected ($10^{7.9}$) in oropharyngeal and cloacal swabs of the control chickens, viral shedding decreased by time in vaccinated chicken groups more than in control .

There was a statistical significant difference among groups, in the 3rd day results revealed a higher rate of virus shed in groups (A1, A2, B1, and B2). the virus shedding decreased in group A3, A4, A5, A6, B3, B4, B5, B6, C1, C2, C3, C4, C5, C6, D1, D2, D3, D4, D5 and D6 lower titers recorded in group D5. Higher titers of challenge virus were detected from tracheal swabs in the SPF ECE, the results were 100% in groups (A1, A2, B1, and B2), while it decreased for the other groups. In the 5th day post challenge, there were higher virus shedding titer in groups A1, A2, A3, A4, A5, B1,B2, lower shedding titers detected in group D6. However, it found by virus isolation the results were 100% in groups A1, A2, A3, A4 ,A5, B1, and B2.By the day 7, results were significantly different with high titer in groups A1, B2, Low titer for the other gps . At the 10th day post challenge, no shedding in group A1,A2,A3,A4 because of death of all chicken in A1,A2,B1,B2.

For cloacal Swabs Higher titers of challenge virus were detected in the SPF ECE, the results were high (A1, A2, A3, B1, and B2), while it decreased for the other groups challenge for the 3rd day. However, it found by virus isolation the results were high at A1, A2, A4, A5 ,B1,B2 for the 5th dpi (day post inoculation) by the day 7, results were significantly different with high titer in groups A1, Low titer in the other g . At the 10th day post challenge, higher titers of challenge virus were detected in A5.

We found that high protection result was for (GroupD) which was vaccinated at twelve day old. Results of viral shedding of (Group D) are described in table (5) and table (6).

The result in table (5showed the results of the shed virus titer after 2wpv (D1), 3 WPV (D3) and 4 WPV (D5) tested for the tracheal and cloacal samples at different times

Table 4 :- Protection results of (Group D) vaccinated with inactivated H5N1 vaccine at twelve day old and challenged with H5N2

Challenge	No of birds	No of died birds/day post challenge										Total deaths	Protection%
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th		
2nd WPV	10				3		2	1	1			7/10	30%
Control	10	2	4	4								10/10	0%
3rd WPV	10		1		1			2				4/10	60%
Control	10	3	2	5								10/10	0%
4th WPV	10			1		1						2/10	80%
Control	10	5	4	1								10/10	0%

Table 5 :- Protection results of (Group D) vaccinated with inactivated H5N1 vaccine at twelve day old and challenged with H5N8

Challenge	**No of birds**	No1st of died birds/day post challenge										Total deaths	Protection%
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th		
2nd WPV	10				1	1		2	1	3		8/10	20 %
Control	10			4	3	2	1					10/10	0%
3rd WPV	10				1		1	2		1		5/10	50%
Control	10				3	3	4					10/10	0%
4th WPV	10					1	1		2			4/10	60%
Control	10			2	4	1	1		2			10/10	0%

Table (6) : Results of chicken groups vaccinated at twelve day old (Group D) challenged with H5N2 Virus

	Day Post challenge															
	3rd				5th				7th				10th			
	T		C		T		C		T		C		T		C	
	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control
D1	$10^{2.1}$	$10^{4.2}$	$10^{1.8}$	$10^{5.2}$	$10^{2.0}$	ND	$10^{1.8}$	ND	$10^{1.8}$	ND	$10^{1.6}$	ND	$10^{1.6}$	ND	$10^{1.4}$	ND
D3	$10^{1.6}$	$10^{3.9}$	$10^{1.4}$	$10^{5.0}$	$10^{1.4}$	$10^{4.0}$	$10^{1.0}$	$10^{6.0}$	$10^{1.0}$	ND	$10^{1.0}$	ND	$10^{1.0}$	ND	$10^{1.0}$	ND
D5	$10^{1.0}$	$10^{4.0}$	$10^{1.0}$	$10^{4.8}$	$10^{1.0}$	$10^{3.8}$	$10^{1.0}$	$10^{4.6}$	$10^{1.0}$	ND	$10^{1.0}$	ND	$10^{1.0}$	ND	$10^{1.0}$	ND

T:-Tracheal, C:- cloacal, Wpv: weeks post vaccination ,D2: sub group challenged at 2wpv,D4: sub group challenged at 3wpv,D6: sub group challenged at 4wpv

Table (7) :- Results of chicken groups vaccinated at twelve day old (GroupD) challenged with H5N8 Virus

	Day Post challenge															
	3rd				5th				7th				10th			
	T		C		T		C		T		C		T		C	
	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control	vaccinated	control
D2	$10^{3.2}$	$10^{5.2}$	$10^{3.1}$	$10^{5.9}$	$10^{3.0}$	$10^{5.9}$	$10^{3.0}$	$10^{5.1}$	$10^{3.1}$	$10^{5.4}$	$10^{2.8}$	$10^{6.1}$	$10^{2.1}$	ND	$10^{1.8}$	ND
D4	$10^{2.0}$	$10^{5.0}$	$10^{2.0}$	$10^{5.6}$	$10^{2.6}$	$10^{4.8}$	$10^{2.0}$	$10^{5.2}$	$10^{2.4}$	ND	$10^{2.1}$	ND	$10^{2.0}$	ND	$10^{1.6}$	ND
D6	$10^{2.0}$	$10^{4.6}$	$10^{1.6}$	$10^{4.0}$	$10^{1.0}$	$10^{3.6}$	$10^{1.0}$	$10^{3.6}$	$10^{1.0}$	$10^{4.9}$	$10^{1.0}$	$10^{4.2}$	$10^{1.0}$	ND	$10^{1.0}$	ND

T:-Tracheal, C:- cloacal , Wpv: weeks post vaccination ,D2: sub group challenged at 2wpv ,D4: sub group challenged at 3wpv ,D6: sub group challenged at 4wpv

(3rd, 5th 7th and 10th) days post challenge. The lowest virus titer (10^1) was recorded at the 3rd and 5th day post challenge for group D5 in addition to group D3 at the 7th and 10th day, while the highest virus titer was ($10^{2.1}$) for group D1 at the 3rd Post challenge.

As presented in table (6) the shed virus titer after 2wpv (D2), 3 WPV (D4) and 4 WPV (D6) tested for the tracheal and cloacal samples at different times (3rd, 5th 7th and 10th) days post challenge, the lowest virus titer ($10^{1.0}$) was recorded at the 5th, 7th and 10th day post challenge for group D 6, while the highest virus titer was ($10^{3.2}$) for group D2 at the 3rd Post challenge.

4. DISCUSSION

In Egypt, vaccination strategy for prevention and control of AI (Avian Influenza) is very important. In our work, the efficacy of AI H5N1 vaccine using SPF chickens at different ages was recorded. The obtained results indicated the used H5N1 vaccine give protection against other subtypes AI H5 Virus. Our results agreed with Atsushi Yasuda et al., (2016) results who proved that the protection against diverse AI H5 viruses belonging to different clades.

Our result also agreed with Ellis et al., (2004) who proved that H5N2 vaccine could face HPAI H5N1virus challenge so it was able to protect chickens from disease.

Our work results agreed with Lee and Suarez (2005) who decided that one dose of homologous H5N1 vaccine was able to give 100% protection and completely prevent viral shedding after lethal dose virus challenge.

The rate of protection percentage after infection with the H5N2 and H5N8 AIV differ according to age of vaccination. All birds of control groups died within 3-7 days. The groups of chicks vaccinated at 1 day old showed the highest mortality rate than the other groups. While the chickens vaccinated at 10 and 12 day-old recorded low mortality and high protection percentage reach (80%). These agreed with Ellis et al., (2004) who proved that, when the chickens were between 9 and 18 days post-vaccination, the infection spread to the recently vaccinated birds, low rate of H5N1 mortality were recorded. While after 18 days post-vaccination, no deaths from H5N1 AI occurred and with intensive monitoring by isolation of the virus from these farms recorded no evidence of virus shedding.

Our results agreed with Beato et al., (2007) who proved that outbreaks of AIV were worldwide and there are many difficulties in controlling this disease.

Vaccination has been advised to limit the economic losses caused by AIV. The use of vaccine containing a heterologous neuraminidase to the field virus is the base of vaccination system in the poultry farms and thus reducing the viral shedding, and clinical symptoms.

Also in this study our results were in agreement with Bublot et al., (2007) who decided that all unvaccinated challenged birds died within 2 days, while the protection percentage of the chickens vaccinated with H5N9WI and H9N9It respectively was 90% and 100%. Cloacal shedding was prevented and oral shedding decreased by vaccination and challenge with Asian HPAI H5N1virus.

5. CONCLUSION

The inactivated AIV H5N1 vaccine provided protection against AIV H5N2 and H5N8 subtypes and decreased virus shedding from the challenged chicken.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

6. REFERENCES

1. Atsushi Y., Motoyuki E., Kristi M. D., Zoltan P., Vilmos P., Darrell R. K. , Yannick G. (2016). Development of Vaccines for Poultry Against H5 Avian Influenza Based on Turkey Herpesvirus VectorDOI: 10.5772/64348
2. Anon (1971). Methods of examining poultry biologics for identification and quantifying avian pathogen. Natl. Acad. Sci., Washington, D.C.
3. Balish A.L. ,C.T. Davis, M.D. Saad, N. El-Sayed, H. Esmat, J.A. Tjaden, K.C. Earhart, L.E.Abd Ahmed, M. El-Halem, A.H. Ali, S.A. Nassif, E.A. El-Ebiary, M. Taha, M.M. Aly, A. Arafa, E. O'Neill, X. Xiyan, N.J. Cox, R.O. Donis, A.I. Klimov (2010).Antigenic and genetic diversity of highly pathogenic avian influenza A (H5N1) viruses isolated in Egypt Avian Dis., 54 (2010), pp. 329-334
4. Beard, C.W. (1989). Influenza. In H.G. Purchase, L.H. Arp, C.H. Domermuth and J.E. Pearson (Eds.), A Laboratory Manual for the Isolation and Identification of Avian Pathogens 3rd ed: 110–113
5. Beato. M.S.; Rigoni, M.; Milani, A. and Capua, I. (2007). Generation of avian influenza reassortant viruses of the H7N5 subtype as potential vaccine candidates to be used in the framework of a "DIVA" vaccination strategy. Avian Dis.Mar; 51(1 Suppl): 479-80.
6. Bouvier and Palese, Bouvie N. M. ,Peter P.(2008). The biology of influenza viruses (Suppl 4):D49-53. doi: 10.1016/j.vaccine.2008.07.039.
7. Boyle, D.B.; Selleck, P. and Heine, H.G. (2000). Vaccinating chickens against avian influenza with fowlpox recombinants expressing the H7 haemagglutinin. .Australian Veterinary Journal 78, 44-48.
8. Bublot, M.; Pritchard, N.; Cruz, J. S.; Mickle, T. R.; Selleck, P. and Swayne, D. E (2007).Efficacy of a fowlpox-vectored avian influenza H5 vaccine against Asian H5N1 highly pathogenic avian influenza virus challenge. Avian Diseases 51: (1), 498-500.
9. Cattoli, G.Drago , A. Maniero, E. Toffan , S. Bertoli , C. Fassina , C. Terregino (2011). . Evidence for differing evolutionary dynamics of A/H5N1 viruses among countries applying or not applying avian influenza vaccination in poultry Sciencedirect.9368-9375
10. Lee, C. W and Suarez, D. L. (2005).Avian influenza virus: prospects for prevention and control by vaccination. Animal Health Research Reviews 6(1): 1-15.
11. LiJun, J.; XiuFan, L.; YanMei, Z.; DaXin, P. and RuKuan, Z. (2004). Effect of rFPV-IFN- gamma as immune-potentiator on active immunization induced by H5 subtype avian influenza vaccines in chickens, J. of Agricultural Biotechnol.; 12(4): 427-430.
12. Ellis T.M. Leung, C.Y. Chow, M.K.Bissett, L.A. Wong, W. Guan, Y. and Peiris M. (2004). Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. 33, 405-412.
13. Hafez A. Arafa , E.L. Elwahab (2010). Avian influenza H5N1 virus infections in vaccinated commercial and backyard poultry in Egypt.Poultry Science 89(8):1609-13
14. OIE, (2010). Terrestrial Animal Health Code, (Chapter 10.4)
15. OIE, Terrestrial Manual Health Code (2015). Chapter 2.3.4 (infection with avian influenza virus).
16. Spackman E., Killian M.L (2014) Avian influenza virus isolation, propagation, and titration in embryonated chicken eggs. Methods Mol Biol 1161:125–140
17. Spearman-Karber (1961).Methods for calculating 50% endpoint using serial dilutions sited in Villegas, P. and Purchase, H.G. 1989. A laboratory Manual for the isolation and Identification of Avian Pathogens, 3rd edition, University of Pennsylv.