Efficacy of a tetravalent oil emulsion vaccine against Avian Infectious Bronchitis and Avian Influenza subtype H9N2 viruses

Elseify. R.M[1].; El-Bagoury. G.F[2].; and Salman. O.G.A[1], Alkhalefa.F. Noura[3]

[1] Newcastle disease department, veterinary serum and vaccines research institute (VSVRI), Abbassia, Cairo, Egypt.
[2]Department of Virology, Faculty of Veterinary Medicine, Benha University, Egypt.
[3]Department of Virology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt.
zn_r@hotmail.com

Abstract: Avian Infectious Bronchitis (AIB) and Avian influenza virus (AIV) H9N2 are worldwide viral diseases leading to heavy economic losses on the poultry industry either each one alone or in mixed infection. Vaccinations considered the main preventive measures for most poultry viral diseases worldwide. In case of Infectious Bronchitis Virus (IBV), vaccination is only partially successful because of the continual emergence of antigenic variants. This study was conducted to evaluate the potency of a tetravalent inactivated vaccine in protection of chickens against currently circulated IBV local Egyptian variant strain and AIV H9N2. AIV H9N2. The prepared inactivated tetravalent IBV (M41, H120 and variant starin) with AIV (H9N2) vaccine using Montanide ISA-70 VG as oil adjuvant was subjected to sterility, safety and potency tests. Vaccine potency evaluation was based on measurement antibody response of vaccinated chickens by HI test for AIV subtype H9N2 and ELISA for IBV in addition to resistance to challenge viruses. The prepared vaccine proofed to be sterile, safe and potent. It stimulated the production of specific antibodies against the IBV as fast as 1st WPV. The maximum level of specific IBV ELISA antibody titers (2314) was observed at the 12th WPV and still high there till the end of the experiment**.** Anti-haemagglutinating antibodies were detected rapidly in 1st WPV against AIV (H9N2) and reached its peak twice, the 1st one was on the 4th WPV while the 2nd was on 8th WPV (9.4 $\log_2$) and then decreased gradually reaching 7.7 $\log_2$ at the end. The vaccinated chickens showed 100% protection against AIV H9N2 and IBV virus, where neither clinical signs nor post mortem lesions were observed.
[Elseify. R.M.; El-Bagoury. G.F.; and Salman. O.G.A and Alkhalefa. F. Noura. **Efficacy of a tetravalent oil emulsion vaccine against Avian Infectious Bronchitis and Avian Influenza subtype H9N2 viruses.** *Life Sci J* 2017;14(5):95-101]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 13. doi:10.7537/marslsj140517.13.

Key words:- IBV, AIV (H9N2), oil inactivated vaccine, HI, and ELISA.

1-Introduction

Avian infectious bronchitis (IB) is an acute, highly contagious, viral disease of chickens, characterized primarily by respiratory signs in growing chickens, while in hens, decreased egg production and quality and it can be nephropathogenic causing acute interstitial nephritis, urolithiasis and mortality **(Cavanagh and Gelb, 2008)**. It is caused by avian infectious bronchitis virus (IBV) a member of the genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*, in the order Nidovirales **(Woo *et al*., 2012)**. IBV infection was firstly reported in Egypt earlier in 1950 by **Ahmed (1954)** and confirmed by **Eissa et al, (1963)**. Recently, many studies were done on the isolation of IBV from chickens suffering from respiratory or renal signs **(Sedik, 2005**) and a new strain of IBV was isolated from 38 day old broiler chick in Beni-Seuf in 1998 and identified using EM, AGP test and RT-PCR **(Abdel-Moneim et al., 2002).**

Avian influenza virus (AIV) subtype H9N2 was firstly detected in United States at 1966 in turkeys and spread to other countries **(Alexander, 2000)**. It is low pathogenic causing slight to moderate mortality with apparent clinical signs characterized by respiratory symptoms, depression, and egg production reduction **(Lee et al., 2000)**. In young chickens when the immunity get lowered, severe respiratory disease with high mortality rate occurred **(Bano et al., 2003)**. AIV subtype H9N2 is a member of the avian influenza virus type A of *Orthomyxoviridae* family **(Swayne and Halvorson, 2003)**. In Egypt, AIV (H9N2) has already detected and sequenced from samples obtained from live birds in 2003 and submitted through NAMMRU3 to SEPRL, USA to complete characterization **(Arafa et al., 2012)**. It was reported in Egypt at first time among broilers and layer breeders by **Hussien and El-Azab (2001).**

Mixed infection of chickens with IBV and Newcastle or Avian Influenza (H5N1 or H9N2) without an effective vaccination program lead to high economic losses **(Hussien et al., 2013)** in addition to occurrence of multiple serotypes of IBV that hinder the efforts to control the disease by immunization **(Cavanagh et al., 1997)**.

The use of oil emulsion (OE) vaccines for prevention and control of avian diseases was rapidly increased and these vaccines stimulate humoral immunity in the host protecting it against viruses. Vaccination of poultry with OE vaccines against AIVs especially mildly pathogenic viruses has been increasing in recent years **(Rajabi, et al., 2010)**.

The increasing emergence of IBV genotypes and lack of cross protective immunity have augmented the pace of interest in the development of novel IBV vaccines. So, the present study aimed to formulate a potent tetravalent inactivated OE vaccine eliciting protection for poultry against different serotypes of IBV including variant type and AIV H9N2 to control both diseases in one shot consequently saving time, effort of labor and avoiding stress of chickens during vaccination.

2-Material and methods

2.1. Viruses:

- Avian infectious bronchitis virus (IBV): three strains of IBV were used, IBV M41 and IBV H120 strains with initial virus titer of $10^{7.5}$ and 10^{8} EID_{50}/ml respectively, kindly provided from Newcastle disease department, veterinary serum and vaccines research institute (VSVRI), Abbassia, Cairo, Egypt, and the 3rd IBV strain was local variant Egyptian Giza-291F-2012 strain kindly obtained from animal health research institute (AHRI) with initial titer $10^{8.2}$ EID_{50} / ml, and used as challenge virus.

- Avian influenza virus (AIV) subtype H9N2: Local Egyptian AIV H9N2 strain A/chicken/Egypt/D4692A/2012, was provided to VSVRI by the National Research Centre (NRC), Environmental Research Division, Egypt. The virus original titer was $10^{9.5}$ EID_{50}/ml and 2^{11} HAU. The nucleotide sequence of the HA gene was submitted into Gen Bank with accession number JX912997, and used as challenge virus.

2.2. Specific pathogen free embryonated chicken eggs (SPF-ECE): It were obtained from Nile-SPF-Eggs Farm, Koom Oshiem, Fayom, Egypt and used for virus propagation, virus titration and assurance of complete virus inactivation.

2.3. Chickens: Twenty one days old SPF chicks were obtained from Nile-SPF-Eggs Farm, Koom Oshiem, Fayom, Egypt. The chicks were housed in brooder units within isolation facilities and reared under complete hygienic condition. Chickens were used for evaluation of the prepared vaccine.

2.4. Vaccine preparation:

2.4. 1. propagation and titration:

It was carried out in SPF-ECEs according to **(Garcia et al., 1998)**. The obtained harvest from virus was titrated in SPF ECEs according to the standard methods described in **FAO (1978)** and the titer was calculated according to the method of **Reed and Muench (1938).**

2.4.2. Virus inactivation (OIE manual, 2004):

It was carried out by applying of 0.1% formalin / liter viral fluid (Sigma-Aldrich Laborcheikalien Gmbh, Germany) with agitation using magnetic stirrer for about 18 - 20 hrs at 25 ^{o}C, and then add 2% of the total fluid volume sodium bi-sulphite to stop formalin reaction and stored at 4^{o}C for vaccine formulation.

2.4.3. Completion of inactivation:

Sample from the inactivated virus was tested for completion of inactivation by inoculation into a five, 10 day old, SPF ECEs. Three successive blind passages were carried out before it was considered completely inactivated.

2.4.4. Preparation of oil emulsion tetravalent vaccine:

The vaccine was prepared in the form of W/O using Montanide ISA 70 VG (SEPPIC, PUTEAUX CEDEX France. Batch number: T34651, Product code: 36018A) according to manufacturer instructions with an aqueous to oil ratio of 3:7, keeping the antigen amount of all the four viruses the same in equal weight and titer

2.4.5 Sterility test:

The prepared vaccine was tested for bacterial and fungal contaminants by cultivation on nutrient agar, nutrient broth and sabouraud dextrose agar.

2.4.6. Safety test:

Twenty (21day old) SPF chicks divided in 2 equal groups. Chick of the first group was injected, S/C at the nap of the neck, with double dose (1 ml) of the prepared vaccine. The 2nd group was left without injection as control. Both groups were observed, daily, for 2 weeks for any clinical signs, local or systemic reaction.

2.4.7. Vaccine potency:

The efficacy testing of the inactivated vaccine was carried out as (Piela, et al 1984). The inactivated vaccine was administered to 20 three-week old SPF chicks of intramuscularly at 0.2 ml per chick. Another 10 three-week old chicks were left unvaccinated. Blood was collected from all chicks before administration of vaccine and subsequently at weekly interval for 16 weeks. The serum separated and heat inactivated at 56^{o}C for 30 min. serum samples were stored at -20 ^{o}C until tested by HI test for AIV subtype H9N2 and ELISA for AIBV in addition to resistance to challenge viruses.

2.5. Experimental design

A total of 100 experimental SPF chickens of 21 days old were divided into 2 groups. The chickens of group (1) were vaccinated S/C with the prepared inactivated tetravalent AIBV and AIV-H9N2 oil emulsion vaccine in a dose of 0.5 ml per chicken,

while the 2nd group was kept as unvaccinated negative control.

It was followed up to16 weeks starting from 0 time. Blood samples (individually from jugular vein of chickens) were collected weekly till the 6th WPV, then every 2 weeks till 12th WPV and finally at 16th WPV. Blood samples collected from SPF chickens without anticoagulant and left to coagulate. Sera were separated and kept at -20°C till used to evaluate humoral immune response through HI test for AIV-H9N2 and ELISA for AIBV.

2.6. Micro Haemagglutination test (HA) and HI test:

It was carried out according to **Allan and Gough (1976)** in a V-bottomed micro titer plate to determine the haemagglutinating titer (expressed as HAU) of virus which collected from SPF ECE after propagation HI test was carried out using 4 HAU of homologous antigen (AIV-H9N2), according to **Majujabe and Hitchner (1977)**, to estimate antibody titers in sera of vaccinated and unvaccinated chickens.

2.8. ELISA

IBV -Ab ELISA kit was supplied by (Affinitech, LTD, Suit 2, Bentonville, catalog no. IBV-0500 and serial no.5548). It was carried out for detection of antibodies against IBV in serum samples post vaccination **(Snyder et al., 1984)**.

2.9. Challenge test:

After 28 days post vaccination, 20 chickens fro vaccinated group were divided into 2 sub-groups A and B (each group contain 10 chickens). Chickens of group A were challenged by intranasal route using AIV H9N2 strain A/chicken/Egypt/D4692A/2012. The challenge dose was 0.1 ml containing 10^6 EID_{50}/ml. The control unvaccinated group (10 chickens) challenged with the same virus. Chickens of group B were challenged by intra-ocular and intranasal route with IBV local variant Egyptian Giza-291F-2012 strain using 10^4 EID_{50}/0.1ml, and the control unvaccinated group challenged with the same virus. The challenged chickens were observed daily for 2 weeks post challenge to detect any clinical signs and deaths.

Tracheal swabs were collected at 2, 4 and 6 day post challenge to detect and determine the virus shedding for both types of viruses by using real time PCR (rRT-PCR) **(OIE, 2008 and Tawfik et al., 2013**).

2.10. Real time PCR:

Viral RNA extraction from tracheal swabs collected from each chickens after challenge either with LPAIV-H9N2 or AIBV using Qiagen® RNeasy Mini kit according to manufacturer's instruction. Real-time RT-PCR reactions were performed at Animal Health Research Institute, using an Applied Biosystems 7500 Fast Real-Time PCR thermo cycler. For detection of the virus shedding after challenge. Briefly 25ul of the reaction mixture consisted of12.5 µl of 2x Quanti Tect Probe RT-PCR Master Mix,0.5 µl Forward primer,0.5µl reverse primer,0.125µl Probe,.25µl Quanti Tect RT Mix,6.125 µl RNase Free Water and 5 µl Template RNA. The thermal program was 40 cycle of 50c for 30 min for c DNA synthesis, denaturation at 94c for30 sec, annealing at 60c for 45 sec and extension 95c for 15 sec.

Table (1): Oligonucleotide sequence used for detection of IBV and AIV after challenge using real time PCR

Virus	*Gene*	*Primer/ probe sequence 5'-3'*	*Ref*
AIV (H9N2)	H	H9F 5'GGAAGAATTAATTATTATTGGTCGGTAC 3'	**Ben Shabat et al., 2010**
		H9R 5'GCCACCTTTTTCAGTCTGACATT 3'	
		H9 Probe 5'AACCAGGCCAGACATTGCGAGTAAGATCC3'	
AIBV	N	AIBV-fr 5'ATGCTCAACCTTGTCCCTAGCA3'	**Meir et al., 2010**
		AIBV-as 5'TCAAACTGCGGATCATCACGT3'	
		AIBV-TM 5'TTGGAAGTAGAGTGACGCCCAAACTTCA3'	

3. Results and Discussions

Co-infection of Avian Influenza Virus Subtype H9N2 and Infectious Bronchitis is one of the most mixed viral infections in broiler chickens with respiratory disease problems in Egypt **(Hassan et al., 2016)**.

Vaccines intended to protect against respiratory symptoms and drop of egg production or quality and it may contain one or more virus. Important considerations include the selection of viruses, but equally the question what purpose the vaccine shall serve? So a trial of vaccine formulation containing

AIV (H9N2) and IBV was performed. The Massachusetts (M41) strain, HI20, and other vaccines of the Massachusetts serotype are used widely around the world so IBV strains (M41, H120 and variant) were selected representing the antigenic spectrum of isolates in Egypt to combat most of IBV strains.

Our study was designed to prepare a tetravalent inactivated OE vaccine to induce protection against H9N2 AIV and IBV. Vaccine was prepared as W/O emulsion and its evaluation was based on humoral immune response and ability to prevent clinical signs and virus replication in oropharynx (shedding). HI test is the test of choice for estimation of antibody titer for AI vaccination (OIE, 2014).

AIV (H9N2) and the 3 strains of IBV were propagated in 10 days old SPF-ECEs through allantoic cavity revealing infectivity titer of 10 log10 EID50/ml for AIV, 10 log10 EID50/ml for IBV Strain H120 and 9.5 log10 EID50/ml for IBV Strain M41 and 8.2 log10 EID50/ml for IBV Variant Strain (Table 2).

The viruses were propagated for detection of active virus. Complete inactivation of the harvested AIV (H9N2) fluid was achieved after 18 hours incubation with formalin at a final concentration of 0.1 %, where neither HA activity nor death of embryos were noticed in the inoculated 10 days old SPF-ECE with the 3rd passage from inactivated AIV (H9N2) also the harvested fluid of the 3 strains of IBV were completely inactivated where neither embryonic lesions (dwarfism) nor deaths were occurred in the inoculated 10 days old SPF-ECE with the 3rd passage from inactivated IBVs.

Montanide ISA-70 VG was used in the vaccine as an immune-stimulating agent in a ratio of (7:3) Wt/Wt as recommended by its producer. The prepared inactivated tetravalent AIB and AIV (H9N2) vaccine was cultured on different synthetic media for detection of bacterial and fungal growth. It was found that, the vaccine was completely free from any bacterial and fungal contaminants.

The prepared vaccine was injected into each of ten 21 day old SPF chickens through S/C route in a double dose (1ml/bird) at the nap of the neck and observed daily for 21 days. No abnormal local or systemic reaction occurred and this was a proof to the safety of the prepared vaccine. These are coming parallel to the recommendation of **(OIE, 2014)**.

Blood samples were collected weekly up to 6th WPV, then biweekly till 16th WPV, from vaccinated and control birds and sera were separated for measuring the antibodies either by ELISA for IBV or HI for AIV (H9N2).

The vaccine stimulated the production of specific antibodies, in the vaccinated birds, against the IBV as fast as 1st WPV that detected by ELISA as shown in table (3) where it was 390 for vaccinated group and 17 for control one. Gradual increase in the ELISA antibody titers was observed throughout the 16 weeks of monitoring period. The maximum level of specific AIBV ELISA antibody titers (2314) was observed at the 12th WPV and still high till the end of the experiment. Evaluation of humoral immune response against AIV (H9N2) was carried out using HI test as assessed by **Adrianus and Richard, 2009** where they described the HI test as the benchmark for assessing the antigenic relationships between influenza viruses and post infection serum. Also **Swayne et al., 1998** proofed that the haemagglutinin is the major influenza protein that elicited a protective immune response and antibodies directed against it are readily detected and quantified serologically by subtype specific HI test. Anti-haemagglutinating antibodies were detected rapidly in 1st WPV against AIV (H9N2) for vaccinated group and reached its peak twice, the 1st peak was on the 4th WPV while the 2nd was on 8th WPV (9.4 log2) (table 4) and then begin to decrease gradually reaching 7.7 log2 at the end.

Concerning the virus shedding of AIV from challenged vaccinated and unvaccinated chicken groups by real time PCR, it was found that after 2, 4 and 6 days post challenge of group A with H9N2 strain, no viral shedding was detected while shedding of virus was detected in oropharyngeal swabs that collected from control group that challenged with the same virus as it was shown in table (5). That fulfill with **(OIE, 2012)** concerning the evaluation of vaccines by challenge test where to evaluate the efficacy of vaccine, it must be reduce or prevent the shedder virus compared with positive control group.

Also it was found that after 2, 4 and 6 days post challenge of group B with IBV strain, no detection of viral shedding while shedding detected in the control group that challenged with the same virus as shown in table (6).

In table (7) showed that vaccinated chickens showed 100% protection level against AIV and IBV virus, where this groups showed no clinical signs in comparison to unvaccinated control groups that showed slightly respiratory signs with depression and decrease in feed intake with slight congestion in lung, liver and kidney in post mortem lesion. Inactivated variant vaccines may offer better protection against challenge with the virulent live variant IBV than inactivated vaccines containing standard serotypes such as Massachusetts and Connecticut **(Ladman et al., 2002)**.

Depending on the for mentioned results, it could be concluded that the prepared inactivated tetravalent IBV and AIV (H9N2) vaccine could be used safely in immunization of chickens against AIB and AIV H9N2

achieving double protection for both viruses as well as broad spectrum protection against IBV strains.

Table (2) Titers of the 4 viruses used for the vaccine preparation

Virus	Titer Log 10 EID_{50}/ml
Avian influenza virus (H9N2)	10
Infectious bronchitis virus (H120)	10
Infectious bronchitis virus (M41)	9.5
Infectious bronchitis variant strain	8.5

Table (3) Mean ELISA antibody titers of the different groups immunized with different vaccines against Infectious bronchitis viruses.

Weeks post vaccination	ELISA antibody titers	
	Vaccinated Group	Control Group
1	390	17
2	796	23
3	1202	56
4	1365	64
5	1495	84
6	1676	91
8	1995	120
10	2280	114
12	2314	122
14	2310	123
16	2305	128

Sample with antibody titers ≥ 400 considered positive sample according to leaflet of Ab ELISA kit that supplied by Affinitech.

Table (4) Mean Haemagglutination inhibition antibody titers in vaccinated group with H9N2 and unvaccinated control group

Weeks post vaccination	Mean HI titer (Log 2)	
	Vaccinated group	Control group
1	5.6	0
2	7	0
3	8	0
4	9.4	0
5	9.2	0
6	8.2	0
8	9.4	0
10	7.9	0
12	8	0
14	7.8	0
16	7.7	0

Table (5): Real time PCR for detection of AIV shedding after challenge

Types of groups		Vaccinated group (A)			Control group		
Days post challenge		2	4	6	2	4	6
Results of Rt-PCR	C.T	NO	NO	NO	23.01	25.23	27.43
	Shedding amount	NO	NO	NO	3.723×10^6	7.862×10^5	2.673×10^5

Note: C.T (Cycle Threshold) RT-PCR (Real time polymerase chain reaction)

Table (6): Real time PCR for detection of IBV shedding after challenge

Types of groups		Vaccinated group (B)			Control group		
Days post challenge		2	4	6	2	4	6
Results of Rt-PCR	C.T	NO	NO	NO	25.72	19.54	24.09
	Shedding amount	NO	NO	NO	6.928×10^3	7.9×10^6	3.152×10^5

C.T (Cycle Threshold) RT-PCR (Real time polymerase chain reaction)

Table (7): Potency test

Group	Virus used	No. of birds	Total morbidities and mortalities	Protection %
Vaccinated group A	H9N2	10	0	100%
Control of group A		10	6	
Vaccinated group B	IBV	10	0	100%
Control of group B		10	3	

Competing interests

The authors have declared that no competing interests exist.

Correspondence
Name: Elseify. R.M,
Address: Newcastle disease department, veterinary serum and vaccines research institute (VSVRI), Abbassia, Cairo, Egypt.
E-mail: yazzn_r@hotmail.com

References

1. Abdel-Moneim, A.S.; Madbouly, H.M.; Gelb, J.Jr. and Landman, S. (2002): isolation and identification of Egypt/Beni-Suif/01 a novel infectious bronchitis virus genotype. Vet. Med. J. Giza, 50(4): 1065-1078.
2. Adrianus, C.M. and Richard, J.W. (2009): Antigenic cross- reactivity among H5N1 viruses. Chapter-2. Cited in vaccines for pandemic influenza. Book. edited by Richard W. compans and Walter A. Orenstein, Chapter-2.
3. Ahmed, H.N. (1954): Incidence and treatment of some infectious viral respiratory diseases of poultry in Egypt. *Ph.D. Thesis, Fac. Vet. Med. Cairo University, Giza, Egypt.*
4. Anon (1971): Methods of examing poultry biologics for identifying and quantifying avian pathogens. Nat. Acad. Sci. Washington DC.
5. Arafa A., Hagag N., Erfan A., Mahdy W., El-Hussieny M., Adel A., Nasef S. (2012): Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. Virus Genes 45: 283-294.
6. Bano, S. Naeem, K. and Malik, S. A. (2003): Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. *Avian Dis, 47: 817-822.*
7. Ben Shabat, M.; Meir, R.; Haddas, R.; Lapin, E.; Shkoda, I. Raibstein, I. Perk, S. and Davidson, I. (2010): Development of a real-time Taq Man RT-PCR assay for the detection of H9N2 avian influenza viruses. J Virol Methods.; 168 (1-2):72-7.
8. Cavanagh D. and Gelb J., Jr. (2008): Infectious bronchitis. *In:* Diseases of Poultry, Twelfth Edition. Saif Y.M., Fadly A.M., Glisson, J.R., McDougald L.R., Nolan L.K. and Swayne D.E., Eds. Wiley-Blackwell, Ames, Iowa, USA, 117–135.
9. Cavanagh, D., M. M. Ellis. and J. K. A Cook. (1997). Relationship between variation in the SI spike protein of infectious bronchitis virus and the extent of cross-protection. *Avian Pathol. 26:63-74.*
10. Eissa, Y.M.; Zaher, A. and Nafai, E. (1963): Studies on respiratory diseases: Isolation of infectious bronchitis virus. J. Arab. Vet. Med. Ass., 23: 381-389.
11. FAO, Animal Production and Health (1978): Newcastle disease vaccine, their production and use. Series no. 10.
12. Garcia, M., D.J. Suarez J. M. Crawford; J. W. Latimar; R. D. Slemons; D. E. Swayne and M. I. Perdue (1998): Evaluation of H5 subtype avian influenza A viruses in North America. Virus Res. 51, 115-124.
13. Hassan, K.E.; Shany, S.A.; Ali, A.; Dahshan, A.H.; El-Sawah, A.A. and El-Kady, M.F. (2016): Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poult Sci. 1; 95(6):1271-80.
14. Hussein A.; Arafa, A. and Rohaim M. (2013): Sequence analysis of infectious bronchitis virus (IBV) IS/1494 like co-infected with Newcastle disease virus (NDV) in broiler chickens with high mortalities in Egypt 2012. World Veterinary Poultry Association French branch (GF-AMVA) 19th - 23rd August 2013.

15. Hussien H. A. and A. El Azab (2001): Evidence for the presence of H6 and H9 among broiler and layer breeders in Egypt. Proce. XII. Int. Cong. World. Vet. Poultry Assoc. Cairo- Egypt, 227-235.
16. Lee, C. W., C. S. Song, Y. J. Lee, I. P. Mo, M. Garcia, D. L. Suarez, and S. J. Kim. (2000): Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the pathogenic potential of isolate MS96. Avian Dis. 44:527–535.
17. Ladman B.S., C.R. Pope, A.F. Ziegler, T. Swieczkowski T, C.J. Callahan, S. Davison S and J. Jr. Gelb J Jr. (2002): Protection of chickens after live and inactivated virus vaccination against ch11enge with nephropathogenic infectious bronchitis virus. PA/Wolgemuth/98. Avian Dis. 46:938-44.
18. Majujabe, K. A. and Hitchner, S.B. (1977): Antibody response to strain combination of Newcastle disease virus measured by haemagglutination inhibition test. Avian Dis., 21:576-584.
19. Meir, R.; Maharat, O.; Farnushi, Y. and Simanov, L. (2010): Development of a real-time Taq Man® RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. Journal of Virological Methods, 163: 190–194.
20. OIE manual (2004): highly pathogenic avian influenza chapter 2-71.
21. OIE, Tamura (2006): Upbate on avian influenza in animals (type h5). 7 April 2006.Availablefromhttp://www.oie.int/downld/AVIAN%20INFLUENZA/AAIAsian.htm. Accessed 15 September 2006.
22. OIE (2008): Avian infectious bronchitis. OIE Terrestrial Manual 2008. Pp 443-455. Office international des epizootic Paris. Chapter 2.3.2.
23. OIE Manual, (2012): Avian influenza Manual of Diagnostic Tests Chapter 2.3. 4.
24. OIE (2014): Avian Influenza. OIE manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.3.4.
25. Piela, P.H., Gulka, C.M., Yates, V.J. and Chang, P.W. (1984) heterologous Use of egg yolk in serological tests (ELISA and HI) to detect antibody to Newcastle Disease, Infectious bronchitis and Mycoplasma gallisepticum. Avian Dis., 28: 877-883.
26. Rajabi, Z., H.Tayefi-Nasrabadi, A.B.Syofi-Khojin (2010): Evaluation the Quality of Oil-Emulsion Avian Influenza Subgroup H9N2 Vaccines in *In-Vitro*. Procedia in Vaccinology, 2: 22–25.
27. Reed, L. J. and Mennch, H (1938): Simple method for estimating 50 percent and point, Amer. J. Hyg; 27: 793-799.
28. Sediek, M.M. (2005): Studies on infectious bronchitis in chickens. M.V.Sc, thesis, poultry Dis, Fac. Vet. Med. Alex Univ. Egypt.
29. Swayne, D. E., and D. A. Halvorson (2003): Avian influenza: Cited after saif, Y. M.; H. J. Barnes; J. R. Gilson; A. M. Fadly; I. R. Mc-Dougald. and D. E. Swayne 11th ed. of disease of poultry. Iowa State University press Iowa, USA., 135-160.
30. Swayne, D.E.; Senne, D.A. and Beard, C.W. (1998): Influenza, in: Swayne, D.E.; Glisson, J.R.; Jackwood, M.W.; Pearson, J.E. and Reed, W.M. (Eds), Isolation and identification of avian pathogens:150-155. Kennet Square: American Association of Avian Pathologist.
31. synder, D.B., Marquardt, W.W., Mallinson, E.T., Savae, P.K. and Allen, D.C. (1984): Rapid serological profiling by enzyme - linked immunoasorbent assay, III. Simultaneous measurements of antibody titers to infectious bronchitis, infectious bursal disease and Newcastle disease viruses in a single serum dilution. Avian Dis. 28:12-24.
32. Tawfik, H.I.; Salama, E.; Hassan, M. O. and Ahmed, A. (2013): Preparation and evaluation of live bivalent infectious bronchitis vaccine in chicken. Researcher. 5(3): 31-35.
33. Woo P.C.Y., Lau S.K.P., Lam C.S.F., Lau C.C.Y., Tsang A.K.L., Lau J.H.N., Bai R., Teng J.L.L., Tsang C.C.C., Wang M., Zeng B.-J., Chan K.-H. & Yuen K.-Y. (2012): Discovery of seve novel mammalian and avian coronaviruses in the genus *Deltacoronavirus* supports Bat coronaviruses as the gene source of *Alphacoronavirus* and *Betacoronavirus* and avian coronaviruses as the gene source of *Gammacoronavirus* and *Deltacoronavirus. J. Virol.,* 86, 3995–4008.

5/25/2017