Evaluation of combined inactivated vaccines against avian influenza subtypes [H5N1, H9N2] and avian infectious Bronchitis in chicken using montanide and paraffin oil adjuvants

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A B S T R A C T

The goal of the presented work was to prepare and evaluate physical characters and immunogenicity of a combined inactivated Avian Influenza Virus (AIV) H5N1, H9N2 subtypes and Infectious Bronchitis Virus (IBV), strain D-88 oil emulsion vaccines. The prepared vaccines were sterile and safe. Also, they were ensured to be water in oil (W/O) emulsions using drop test, conductivity (zero mS/cm). Particle size were 950 nm and 1050 nm and dynamic viscosities were 19.52 Mpa.s and 39.65 Mpa.s for the vaccine with Montanide™ ISA 71 RVG adjuvant and the vaccine with paraffin oil adjuvant, respectively. They showed stability for 24 months at 4 °C with no separation. Combined inactivated oil emulsion vaccines induced cellular and humoral immune responses in vaccinated chicks. The vaccine with Montanide™ ISA 71-RVG adjuvant provided 100% protection percent for AIV H5N1 without shedding of AIV H9N2 and IBV in comparison to the combined vaccine with paraffin oil adjuvant that gave 84% protection percent for AIV H5N1 and shedding of AIV H9N2 and IBV occurred 6th day post challenge. Montanide™ 71-RVG adjuvant has the flexible ratio of oil and antigenic media in the vaccine (60:40) allowed using large amount of virus that showed good impact on its immunogenicity, over the paraffin oil adjuvant which has restricted ratio of oil and antigenic media of the vaccine (73:27).

Keywords: AIVs, H5N1, H9N2, IBV, inactivated oil-emulsion vaccine.

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1. INTRODUCTION

Avian influenza virus (AIV) and infectious bronchitis virus (IBV) are important viral pathogens in commercial poultry flocks in Egypt causing respiratory manifestations and massive mortalities (Kayali et al., 2016; Kaoud, 2017; Heba et al., 2016). Avian influenza (AI) is a viral disease caused by type A influenza viruses belonging to family Orthomyxoviridae (Olsen et al., 2006).

The virus particle has an envelope with glycoprotein projection with haemagglutinin (HA) and neuraminidase (NA) activity. These two Surface antigens are the basis of the serologic identity of the influenza virus using the letters H and N with the appropriated numbers in the virus designation. There are 16 HA and 9 NA antigens described among the type (A) influenza virus (Fouchier et al.,

2005; Dugan et al, 2008). AIV can be classified into two categories; Low Pathogenic Avian Influenza Virus (LPAIV) and High Pathogenic Avian Influenza Virus (HPAIV), based on the severity of illness caused in domestic birds (Capua and Alexander, 2004). HPAIV may cause up to 100% mortality as subtypes H5 and H7, although not all viruses of these subtypes cause HPAIVs, on the other hand, LPAIVs cause mild to moderate infectious in various domestic and wild bird specious (Mo et al., 1997; Alexander, 2000).

Various subtypes of AIVs H5N1 and H9N2 are circulating among poultry flocks causing severe disease outbreaks with high morbidity and mortality (Cameron et al., 2000; Xu et al., 2007; OIE 2008; Nagarajan et al., 2009; Abbas et al., 2010; Jeong et al., 2010; Kim et al., 2010).

In Egypt, the HPAIV (H5N1) had emerged since February 2006 as the cause of sever disease and high mortality in chickens in production farms and village-based production and causing a great hazard to humans (Aly et al., 2006).

AI H9N2 viruses are characterized as low pathogenic viruses among multiple avian species in Asia, the Middle East, Africa and Europe (Guo, 2002). In Egypt, AIV H9N2 was isolated in December 2010 to May 2011 in chickens, quails and turkeys (Abdel-Moneim et al., 2012a and Arafa et al., 2012a,b). The co-infection of H9N2 with H5N1 was also reported in many cases in poultry in Egypt (Arafa et al., 2012b; Monne et al., 2013; Kayali et al., 2014). Also, some strains of IBV were isolated from several broiler flocks during the H9N2 outbreaks (Nouri et al., 2003; Shapouri et al., 2004; Haqshenas et al., 2005).

Infectious bronchitis virus (IBV) causes a highly contagious respiratory disease and some nephropathogenic strains cause nephritis with the result of significant mortalities in commercial young chickens (Liu and Kong, 2004). It also causes decrease in egg production and egg quality in layers and breeders (Gelb et al., 1991). IBV belonged to genus Gammacorona virus; family Coronaviridae (Carstens, 2009). More than 20 serotypes of IBV were distributed worldwide (Sjaak de Wit et al., 2011). In Egypt, IBV strains have been isolated from chicken flocks (Abd El Rahman et al., 2015). Although these isolates of IBV varied genotypically, they were similar to Massachusetts, D3128, D274, D08880, 793B (4/91 and CR88), IS/885/00 and Egypt/Beni-Suef/01 strains (Sultan et al., 2015). However, variant strains of IBV, Egy/Var- Ⅱ (Ck/Eg/BSU-2, 3/2011), were resembling IS/885/00 strain based on sequence of the HVR-3 of S gene (Abdel-Moneim et al., 2012b).

Vaccination is one of the most important control measures against AIVs H5N1 and H9N2 and IBV (Peyre et al., 2007; Sultan et al., 2004; El-Mahdy et al., 2010). Quality of the adjuvant has direct impact on safety and efficacy of the vaccine, so good physical properties of the inactivated oil emulsion vaccine could in turn increase the immune response to this vaccine. High quality oil emulsion vaccine should be stable, with low viscosity to ease injectability and produce suitable antibody titer in vaccinated birds (stone et al, 1983).

The present study was designed to formulate multivalent inactivated H5N1 and H9N2 AIVs and IBV oil emulsion vaccine using different adjuvants as Montanide™ ISA 71R VG and Paraffin which would be reflected on the immunological response.

2. Materials and methods

2.1. AI Virus strains:

AI (H5N1) vaccinal strain:

The Highly pathogenic (HP) Reasortant Avian Influenza Virus ($H_5 N_1$) subtype Egypt/ Re-1&2 strains seed virus obtained from National Research Center (NRC), [A/chicken/ Q1995D/2010 (strain-1) and A/duck/M2583A/2010 (strain-2)]. The virus strains were egg adapted for 8^{th} passage on specific pathogen free-embryonated chicken egg (SPF-ECE) with HA titer 2^{10} /50 µl and infectivity titer 10^9 EID50/0.1ml (OIE manual 2004). It was used as the seed virus for vaccines preparation.

AI H5N1challenge strain:

Virulent strain of highly pathogenic avian influenza virus (HPAIV) H5N1 subtype (A/Chicken/Egypt/1063/2010) obtained from NRC with infectivity titer of 10^5 EID_{50}/ml used for challenging of vaccinated chicks and are kept under observation for 2 weeks.

AI (H9N2) virus strain:

The low pathogenic Avian Influenza Virus (LPAIV) H9N2 subtype (A/chicken/Egypt/D4692A /2012 obtained from NRC, Egypt. The virus was isolated from chicken farms at Dakahlia governorate and propagated on SPF chicken eggs with original titer $10^{9.5}$ EID_{50} / ml and 2^{11} HA activity (OIE manual 2004). It was used as the seed virus for vaccines preparation and also for challenging of vaccinated chicks.

Infectious bronchitis (D-88) Vaccinal strain:

Local Nephropathogenic strain IBV was isolated from IBV-vaccinated broiler chickens 24 day old at Dakahlia, with a history of respiratory and renal signs (Abdel-Moneim et al., 2006). The local isolate was matched for 96% with isolated strain [Egypt/F/03strain], with accession No. DQ487085 (NCBI) the isolated strain titer was 10^6 EID50/ml. It was kindly obtained from NRC, Egypt.

Infectious bronchitis (M41) vaccinal strain:

The Infectious bronchitis virus (IBV) as Massachusetts M41 strain supplied by department of animal science and agriculture biochemistry, university of Delaware, New York, USA. The Master seed with original titer $10^{10.5}$EID50/ml. The virus used for challenging of chicks against (local Nephropathogenic IBV strain-Egypt/F/03strain-NCBI). The challenge virus dose was adjusted to 0.1ml containing 10^4 EID_{50}/ml /bird *(OIE, 2008; Tawfik et al., 2013).*

2.2. Embryonated Chicken Eggs (ECEs):

Specific pathogen free embryonated chicken eggs (SPF– ECEs) were purchased from the specific pathogen free egg project, Kom Oshim, El-Fayoum Governorate. The eggs were incubated at 37°C and 80% humidity until inoculated at 9-11 days of age via allantoic sac route. They were used for propagation & titration of the seed influenza viruses used for preparation of the vaccinal patches and testing the safety of prepared inactivated virus suspensions.

2.3. Experimental chicks:

Two hundred and ten (210), one-day-old chicks were purchased from specific pathogen free poultry project, Kom Oshim, EL-Fayoum Governorate. They were floor reared, fed on commercial poultry ration, and kept under strict hygienic measures throughout the experiment. The chicks were used for studying the safety and evaluating of the prepared vaccines.

2.5. Vaccine formulation:

Propagation of the Virus in SPF-ECEs:

AI Virus [H5N1, H9N2] strains propagation in embryonated chicken eggs were applied according to Garcia et al. (1998), while IB virus according to OIE, (2008).

Virus Titration in Embryonated Chicken Egg:

Estimation EID50 of the viruses used were calculated according to Reed and Meunch (1938). Titer of AIV (H5N1) was 9.5 log10 EID50/ml and 10.5 log2 HAU/50µl, while AIV (H9N2) titer was 10 log10 EID50/ml and

11 log2 HAU/50µl, using infectivity titration and HA test respectively. Also, titer of IBV was 8.2 log10 EID50/ml using infectivity titration test.

Rapid Plate Hemagglutination (HA) Test:

It was carried out according to the standard method described by Anon (1971)*Virus*

Inactivation of Viruses:

Formalin working solution, HCHO, 37% Analar, BDH. it was diluted in saline in concentration 0.1% for AIVs (OIE manual 2004), while in IBV the final concentration was 0.01% of the total volume (Beard, 1989)**.**

Vaccine preparation:

Inactivated Combined vaccine with Montanide™ ISA 71-RVG oil adjuvant:

A combined vaccine was prepared as water in oil (W/O) emulsion by mixing equal weights from the inactivated AIV H5N1, AIV H9N2 and IBV then this aqueous phase was mixed with Montanide™ ISA 71 RVG oil adjuvant in a ratio of 40:60 weight per weight (Ben Arous et al., 2013).

Inactivated Combined vaccine with paraffin oil adjuvant:

A combined vaccine was prepared as water in oil (W/O) emulsion by mixing equal weights from the inactivated AIV H5N1, AIV H9N2 and IBV, with both the oil soluble surfactant (span 80) and the aqueous soluble surfactant (tween 80) were added to the oil phase (Paraffin oil), (Daoud et al., 2002). This method was modified through addition of aluminum stearate (El-Sayed, 2014), then the aqueous-phase (Inactivated virus suspension) was added to the oil-phase at a ratio 1:3 and the hydrophilic lipophilic balance (HLB) was adjusted to 7.0 as described by Schick, (1966) and Stone, (1988).

2.6. Quality control of the prepared vaccines:

Sterility test:

Experimental batches of the prepared vaccines were tested for sterility and freedom from any fungal or bacterial contaminants by culturing on specific media (Saburaoud glucose agar searching for fungus contamination after incubation at 25°C for 14 days, Nutrient agar media and Thioglycolate broth searching for aerobic and anaerobic bacterial contamination, respectively after incubation at 37°C for 72 hours).

Safety test:

Two groups, each of 10 chicks of 3 weeks old were inoculated with 2 field doses (1ml) of the prepared vaccines at the nap of the neck in addition to a control non-vaccinated group. The vaccinated chicks were observed for 2 weeks for any signs of local reaction or appearance of any clinical signs. After 5 days of inoculation, some birds were subjected to post mortem examinations to detect any pathological lesions.

Physical stability:

Physical properties of the emulsions were determined as Drop test, Conductivity test, Particle size, Dynamic Viscosity and Real-time test.

2.7. Experimental design:

Physical properties of the prepared vaccines:

They were determined as describe by Brugh et al., (1983) and Stone et al., (1988).

Efficacy of prepared vaccines:

Both prepared combined inactivated AIVs H5N1, H9N2 and IBV vaccine with Montanide™ ISA 71-RVG oil adjuvant and combined inactivated AIVs H5N1, H9N2 and IBV vaccine with paraffin oil adjuvant were chosen regarding to their physical properties and subjected for evaluation of both cell mediated immune response and humoral immune response.

Potency of Prepared vaccines

Challenge experiment was carried out to determine the potency of the prepared vaccines. A total of 450 one-day old SPF chicks were reared under complete hygienic measures in special isolators, at 28 days of age the chicks were divided into 9 equal

subgroups groups each of 50 chicks and treated as follow:

G1: Chicks inoculated with combined inactivated AIVs H5N1, H9N2 and IBV vaccine with Montanide™ ISA 71-RVG as adjuvant. It was divided into three subgroups G1-A **(**Challenged with HPAIV H5N1 strain), G1-B **(**challenged with AIV H9N2 strain) and G1-C **(**Challenged with IBV (M41) strain.

G2: Chicks inoculated with combined inactivated AIVs H5N1, H9N2 and IBV vaccine with paraffin oil as adjuvant. It was divided into three subgroups G2-A **(**Challenged with HPAIV H5N1 strain), G2-B **(**challenged with AIV H9N2 strain) and G3-C **(**Challenged with IBV (M41) strain.

G3: Chicks kept in separate isolators as non-vaccinated control group. It was divided into three subgroups G3-A **(**control group for AIV H5N1), G3-B **(**control group for AIV H9N2) and G3-C **(**control group for IBV).

2.8. Samples:

Whole blood samples:

Jugular blood samples from vaccinated and non-vaccinated chicks were collected with anticoagulant (Heparin 20-40 IU/ml) at 3, 7, 10, 14, 21 and 28 days post vaccinations for lymphocyte blastogenesis assay and phagocytic activity test.

Serum samples:

erum samples were collected from all chicks (vaccinated and non- vaccinated) weekly till 10^{th} week post vaccination then every 2week till the 31^{th} week post vaccination. The sera were inactivated at 56°C for 30 minutes, and then stored at -20°C until used in HI test.

2.9. Evaluation of cellular immune response for prepared vaccine:

Evaluation of lymphocyte transformation:

Separation of lymphocytes, determination of viable cell number, and setting up of lymphocytes was performed depending on the instructions of cell proliferation (XTT) kit (ATCC, USA) and the test was performed according to Scudiero et al., (1988). The test was applied according to the method described by Lucy, (1977) and Lee, (1984).

Evaluation of phagocytic activity of chicken macrophages by using Candida Albicans:

Separation of macrophages by ficol hypaque and cultivation of mononuclear cells were performed according to Richardson and Smith, (1981) and modified by Hussien, (1989) .The percent of phagocytosis and phagocytic index was calculated as follow:

$$phagocytic\ percentage = \frac{no\ of\ phagocytes\ which\ ingest\ Candida}{total\ no\ of\ phagocytes} \times 100$$

$$phagocytic\ index = \frac{total\ no\ of\ phagocytes\ which\ ingest\ more\ than\ two\ Candida}{total\ no\ of\ phagocytes\ which\ ingest\ Candida}$$

Evaluation of the humoral immune response for prepared vaccines using Hemagglutination (HA) and Hemagglutination inhibition (HI) test:

The Hemagglutination (HA) and hemagglutination inhibition (HI) test were carried out following the recommendation of (OIE-Manual, 2004). The used antigens in HI test should be prepared from the pure, well identified homologous AIV H5N1 (A/Chicken/Egypt/1063/2010) and AIV H9N2 (A/chicken/Egypt/D4692A/2012).

Enzyme Linked Immunosorbent Assay (ELISA):

Commercial ELISA kits used for monitoring serum antibody responses against IBV. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges according to *Snyder et al., (1986).* ELISA Kit was obtained from ID.VET, France innovative diagnostic poultry immune assays. ID SCREEN IBV indirect test kit (IBVS ver 0614 GB); Batch No: 949, product code: IBVS.

2.10. Challenge of chicks vaccinated with AI vaccine:

Challenge with HPAIV (H5N1) Strain

Four weeks old chickens (vaccinated and un vaccinated control) were challenged by 0.5 cm /bird by I/M route of virulent strain of HPAIV H5N1 subtype (A/Chicken/Egypt/1063/2010) with infectivity titer of 10^5 EID_{50}/ml. The challenged birds were observed for 15 days and collect serum samples during challenge period, dead birds through this time were recorded and examined for p/m lesions.

$$\text{Protection \%} = \frac{\text{No.of survival}}{\text{total No.of challenge of birds}} X100$$

Challenge with Infectious bronchitis (M41) Vaccinal strain;

Four weeks old chickens (vaccinated and unvaccinated control) were challenged by Infectious bronchitis (M41) Vaccinal strain. Each bird received a dose of 0.1 ml intra-nasal of IBV strain (10^4 EID_{50}/ml) and observed for 15 days after challenge, for clinical signs and postmortem lesion in trachea and kidney. Tracheal and cloacal swabs were collected at 2^{nd}, 4^{th} and 6^{th} days post challenge to determine the virus shedding using (RT-PCR)

Challenge with LPAIV (H9N2) Strain;

Four weeks old chickens (vaccinated and unvaccinated control) were challenged with the LPAI (A/chicken /Egypt/D4692A/2012 H9N2). The birds were inoculated by the intra-nasal (100 µl) of allantoic fluid containing 10^6 EID50 of the virus. Tracheal and cloacal swabs were collected at 2^{nd}, 4^{th} and 6^{th} days post challenge to determine the virus shedding using real time reverse transcription PCR (RRT-PCR).

2.11. *Detection of AIV H9N2 and IBV shedding using RRT-PCR:*

The numbers of viral genome copies were quantified in a TaqMan® real time RT-PCR targeting Influenza (A) hemagglutination gene and IBV nucleoprotein gene using RNA extraction kit (QIA amp Viral RNA Mini Kit, QIAGEN, catalogue No. 52904), real time PCR master mix (QuantiTect probe RT-PCR catalogue no. 204443) and real-time quantification of RNA targets using primers and probes supplied from Metabion (Germany). An absolute quantification was done relatively to a standard curve based on tenfold dilution of an in vitro transcribed RNA template of the challenged virus. The samples were tested using thermal cycling conditions for gene-specific Probe and Primer sets using protocol and methods of Ben Shabat *et al*., (2010) for AIV H9N2 and Meir *et al*., (2010) for IBV. Chickens were sampled and tested on individual basis. Ct value of 40 was selected as the cut-off between positive and negative result and samples with higher Ct were considered as negative for AIV and IBV. This was decided according to the standard curve.

Table 1: Oligonucleotide Primers and probes.

Virus	Gene	Primer/ probe sequence 5'-3'	Reference
H9	H	H9F-GGAAGAATTAATTATTATTGGTCGGTAC H9R-GCCACCTTTTTCAGTCTGACATT H9 Probe [FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[TAMRA]	Ben Shabat *et al*., 2010
IB	N	AIBV-fr-ATGCTCAACCTTGTCCCTAGCA AIBV-as-TCAAACTGCGGATCATCACGT AIBV-TM [FAM]TTGGAAGTAGAGTGACGCCCAAACTTCA [TAMRA]	Meir *et al*., 2010

3. RESULTS

Sterility and safety of the prepared vaccines:

The prepared vaccines were free from aerobic and anaerobic bacteria and fungi. They were completely inactivated as indicated by absence of any pathological lesions, HA activity for AIVs H5N1, H9N2 and/or deaths of inoculated embryos being inoculated in 9 days old, SPF-ECEs through the allantoic sac and candled daily for 6 days. There was no local or systemic reaction and no mortalities among vaccinated chicks indicating safety of both inactivated combined [H5N1, H9N2 and

IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant and inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant.

Assessment of physical characters of the prepared vaccine:

The prepared vaccines were ensured to be water-in-oil (w/o) emulsion type using drop test, this W/O emulsion showed 0 mS/cm conductivity. Inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant showed 19.52 m.pa.s viscosity, 950 µm particle size and long duration of stability equal or more than 104 weeks at +4C° no separation as water release or oil release, while inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant showed 39.65 m.pa.s viscosity, 1050 µm particle size and long duration of for 24 months at +4C° with no separation as water release or oil release as shown in table (2).

Cell mediated immune response:

Lymphocyte blastogenesis showed significant cell proliferation expressed by optical density induced in vaccinated chicks vaccinated by both inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant and inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant from the 3rd day post vaccination (DPV) and increased to reach a maximum value 14th DPV. It was noticed that cell proliferation expressed by optical density induced in chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant showed higher values than that of chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant. All the results were compared with that SPF chicks non-vaccinated kept as negative control that had no lymphocyte proliferation as shown in table (3).

Both phagocytic percent and phagocytic index of macrophages were significantly increased 7th DPV to reach maximum values at 14th DPV in vaccinated chicks both inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant and inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant, when compared with that of non-vaccinated chicks kept as negative control that had no macrophage activity as shown in table (4).

Humoral immune response:

Chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant, showed increased mean log2 HI antibody titer (5.33 log2) against AIV H5N1 from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (10 log2) at the 3rd WPV and remained in suitable levels (4.33 log2) till 31st WPV. Chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant, showed increased mean log2 HI antibody titer (4 log2) against AIV H5N1 from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (8.3 log2) at the 5th WPV, then declined to (4.3 log2) at the 21st WPV then declined gradually to reach the lowest HI antibody titer (1.33 log2) at the 31st WPV. These results were compared with that of non-vaccinated chicks kept as negative control that had no antibody against H5N1 as shown in table (5).

Chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant, showed increased mean log2 HI antibody titer (5.6 log2) against AIV H9N2 from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (10 log2) at the 4th WPV and remained in suitable levels (4.33 log2) till 31st WPV. Chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV]

vaccine with paraffin oil adjuvant, showed increased mean log2 HI antibody titer (3 log2) against AIV H9N2 from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (7.3 log2) at the 4th WPV, then declined to (4 log2) at the 23rd WPV then declined gradually to reach the lowest HI antibody titer (1.0 log2) at the 31st WPV. These results were compared with that of non-vaccinated chicks kept as negative control that had no antibody against H9N2 as shown in table (6)**.**

Chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant, showed increased antibody titer measured by mean ELISA optical density (453) against IBV from the 1st week post vaccination (WPV), then reached the highest values (2108) at the 4th WPV and remained in suitable levels (1204) till 21st WPV. Chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant, showed increased antibody titer measured by mean ELISA optical density (586) against IBV from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (1862) at the 4th WPV, then declined to (542) at the 21st WPV. These results were compared with that of non-vaccinated chicks kept as negative control that had no antibody against IBV as shown in table (7).

Potency of the prepared vaccine:

Both vaccinated and non-vaccinated control chicks were challenged 28 days post vaccination using virulent strains of AIVs H5N1, H9N2 and IBV.

The protection percent against HPAIV H5N1 were 100% in chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant and 84% in chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant, compared with 0% for control non-vaccinated chicks as shown in table (8)**.**

Evaluation of shedding of AIV H9N2 were zero % using RRT-PCR in challenged chicks vaccinated with both inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant and inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant, compared with 100% shedding for control non-vaccinated chicks in tracheal swaps at 2nd, 4th and 6th day post challenge as shown in **t**able (9).

Evaluation of shedding of IBV were zero % using RRT-PCR in challenged chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with Montanide™ ISA 71-RVG oil adjuvant and shedding of IBV using RRT-PCR in challenged chicks vaccinated with inactivated combined [H5N1, H9N2 and IBV] vaccine with paraffin oil adjuvant, were zero % at 2nd day post challenge then shedding occurred at 4th and 6th day post challenge, compared with 100% shedding for control non-vaccinated chicks in tracheal swaps at 2nd, 4th and 6th day post challenge as shown in table (10).

Table 2: physical properties of prepared oil emulsion combined inactivated vaccines against avian AI [H5N1, H9N2] and IBV**.**

Vaccine	Emulsion type		Particle size	Dynamic Viscosity	Emulsion stability per week (real time test)		
	Drop test	Conductivity			25°C	37C°	+4C°
V1	W/O	0 mS/cm	950 µm	19.52 Mpa.s	≤ 64	≥ 13	≤ 104
V2	W/O	0 mS/cm	1050 µm	39.65 Mpa.s	≤ 13	≥ 4	≥104

V1: Combined inactivated vaccine against avian AI [H5N1, H9N2] and IBV Montanide™ ISA 71-RVG.

V2: Combined inactivated vaccine against avian AI [H5N1, H9N2] and IBV Paraffin adjuvant.

Table 3: Lymphocyte proliferation of chickens vaccinated with combined inactivated avian AI [H5N1, H9N2] and IBV oil emulsion vaccines expressed by delta optical density.

Chick group	Cell proliferation expressed by optical density/day post-vaccination					
	3rd	7th	10th	14th	21st	28th
G1	0.9865	1.2435	1.738	2.001	1.1385	0.470
G2	0.760	1.1845	1.532	1.882	0.982	0.3749
G3	0.173	0.198	0.397	0.2452	0.184	0.1025

G (1) Chickens vaccinated by inactivated Combined vaccine with Montanide™ ISA RVG-71 oil adjuvant

G (2) Chickens vaccinated by inactivated Combined vaccine with paraffin oil adjuvant.

G (3): chicks kept as non-vaccinated control.

Table 4: macrophage activity of chicks vaccinated with combined inactivated avian AI [H5N1, H9N2] and IBV oil emulsion vaccines.

Chick group	Phagocytic activities days post vaccination			
	Phagocytic %		Phagocytic index	
	7th	14th	7th	14th
G1	60.7%	86.66%	0.44	0.83
G2	54.54%	78.8%	0.46	0.76
G3	5.26%	3.703%	0.08	0.11

G (1) Chickens vaccinated by inactivated Combined vaccine with Montanide™ ISA RVG-71 oil adjuvant

G (2) Chickens vaccinated by inactivated Combined vaccine with paraffin oil adjuvant.

G (3): chicks kept as non-vaccinated control.

Table 5: mean log2 HI antibody titer against H5N1 of chicks vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines.

Chick Group	Mean log_2 HI antibody titer against H5N1 weeks post vaccination																	
	1	2	3	4	5	6	7	8	9	10	12	15	17	19	21	23	27	31
G1	5.33	7.33	10	9.33	10	9.66	8.66	8.00	8.66	7.66	8.66	7.00	8.00	6.66	7.33	6.00	4.66	4.33
G2	4	4.30	6.60	7.60	8.30	8	7.60	7.30	6.60	7	5.30	4.60	5	4.30	4.30	4	2.66	1.33
G3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 6: mean log2 HI antibody titer against H9N2 of chicks vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines.

Chick Group	Mean log_2 HI antibody titer against H9N2 weeks post vaccination																	
	1	2	3	4	5	6	7	8	9	10	12	15	17	19	21	23	27	31
G1	5.6	7.66	9.33	10	9.66	8.66	9.66	8.33	7.33	8.33	8.33	8.33	8.66	6.66	7.00	7.66	6	4.33
G2	3.000	4.60	6.60	7.30	7.10	6	5.60	6	6.30	6.60	7	5.30	4.60	4.60	5	4	2.660	1.000
G3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 7: ELISA antibody titers against IBV expressed as optical density of chicks vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines.

Chick Group	Mean antibody titer expressed of IBV as optical density weeks post vaccination														
	1	2	3	4	5	6	7	8	9	10	12	15	17	19	21
G1	453	894	1080	2108	1852	1676	1678	1140	1554	1516	1246	1317	1482	1420	1204
G2	568	870	1270	1862	1680	1540	1679	1177	1440	1468	1197	1154	1080	940	542
G3	15	26	48	56	62	86	80	68	76	70	66	58	46	28	32

Table 8: Protection percent in chicks vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines after their challenge with virulent strain of (HPAIV) H5N1.

Chick groups	Number of chicks			Protection Percent
	Challenged	Dead	Live	
G1	50	0	50	100%
G2	50	8	42	84%
G3	50	50	0	0%

Table 9: Evaluation of virus shedding after challenge with AIV H9N2 of chicks vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines using RRT-PCR.

Day post challenge	Group	Result	CT	Shedding amount (Copies)
2nd day	1-B	Negative	No Ct	-
	2-B	Negative	No Ct	-
	3-B	Positive	26.23	3.854 x 102
4th day	1-B	Negative	No Ct	-
	2-B	Negative	No Ct	-
	3-B	Positive	23.15	5.278 X 105
6th day	1-B	Negative	No Ct	-
	2-B	Negative	No Ct	-
	3-B	Positive	24.64	2.377 x 103

G1-B chicks are vaccinated with Combined Vaccine against H9N2 montanide71ISA RVG

G2-B chicks are vaccinated with Combined Vaccine against H9N2 paraffin oil adjuvant

G3-B chicks kept as non-vaccinated control.

Table 10: Evaluation of virus shedding after challenge with IBV of chicks vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines using RRT-PCR.

Day post challenge	Group	Result	CT	Shedding amount (Copies)
2nd day	1-C	Negative	No Ct	-
	2-C	Negative	No Ct	-
	3-C	Positive	22.89	4.832×10^7
4th day	1-C	Negative	No Ct	-
	2-C	Positive	25.314	3.543×10^8
	3-C	Positive	30.14	6.330×10^8
6th day	1-C	Negative	No Ct	-
	2-C	Positive	23.72	2.664×10^3
	3-C	Positive	23.15	5.278×10^5

G1-C chicks are vaccinated with Combined Vaccine against IBV with montanide71-ISA RVG

G2-C chicks are vaccinated with Combined Vaccine against IBV with paraffin oil adjuvant

G3-C chicks kept as non-vaccinated control.

4. DISCUSSION

Inactivated AIVs H5N1, H9N2 and IBV (D-88) were used as the seed virus for vaccine preparation (OIE, 2008). Vaccines were prepared in the formula of water -in oil (W/O) emulsion using paraffin oil (Daoud et al., 2002; El-Sayed, 2014) and using Montanide™ ISA 71 RVG adjuvant ((SEPPIC, France). Quality control of adjuvant emulsion has direct impact on the efficacy and the safety of vaccine. Physiochemical characterization of an emulsion can be defined by various parameters such as droplet test, conductivity, viscosity, particle size and stability at various temperatures. W/O emulsion requires high shear homogenization to get stable formulation (Salager 2000: Lissant 1984).

Drop test showed that the prepared combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines using either Montanide™ ISA 71R VG and Paraffin oils as adjuvants were W/O emulsion type, and conductivity which measured by (mS/cm) unit equal (zero) as shown in table (2). These results were agreed with (Salager 2000: Lissant 1984).

Particle size for the prepared vaccines were 950 nm and 1050 nm and dynamic viscosities were 19.52 Mpa.s and 39.65 Mpa.s for combined inactivated AIVs H5N1, H9N2 and IBV vaccine with Montanide™ ISA 71R VG adjuvant and combined inactivated AIVs H5N1, H9N2 and IBV vaccine with Paraffin oil adjuvant, respectively. These results indicate fast flow time and easy injectability of the prepared vaccines and were agreed with Stone, (1991) and Seppic, (2012) who showed that ideal particle sizing between 0.05µm and 1000µm and was also like the results of the European pharmacopeia, (2010) which stated that acceptance limits of viscosity of vaccine emulsion ranged between $12 \geq R \leq 56$ mpa.s.

The prepared vaccines were stable soon after preparation using centrifugation test. They also were stable for 104 weeks at 4°C using real time test. These results came in accordance with Ben Arous et al., (2013) and El-Sayed, (2014). Also, were similar to the results of Lissant (1984), Stone, (1991) and Salager (2000).

The prepared combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccines were completely sterile with no bacterial or fungal contaminants when tested

on specific bacteriologic and fungal media. In addition, absence of local and systemic reactions and no mortalities were recorded in inoculated chicks which denoted to the safety of the prepared vaccines. Results of the vaccines sterility and safety came in parallel with the recommendations of OIE, (2004).

Evaluation of the potency of both combined inactivated oil emulsion vaccines using either Montanide™ ISA 71R VG and Paraffin oils as adjuvants were carried out in vaccinated chicks through measuring cellular and humoral immune responses and protection percent after their challenge.

Lymphocyte blastogenesis showed significant cell proliferation expressed by optical density induced in vaccinated chicks vaccinated by both inactivated combined vaccines with Montanide™ ISA 71-RVG oil adjuvant and paraffin oil adjuvants from the 3rd day post vaccination (DPV) and increased to reach a maximum value 14th DPV. It was noticed that chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant showed higher values than that of chicks vaccinated with paraffin oil adjuvant (table 3). Both phagocytic percent and phagocytic index of macrophages were significantly increased 7th DPV to reach maximum values at 14th DPV in vaccinated chicks with both Montanide™ ISA 71-RVG oil adjuvant and paraffin oil adjuvant (table 4). These results came in agreement with that of (Madkour 1992) who clarified clearly that chicken vaccinated with oil emulsion vaccine greatly stimulated the cellular immune response as estimated by lymphocyte proliferation test. The results also showed that values of cellular immune response at later stages came in agreement with that of Timms and bracemell, (1983) who stated that once the humoral immune response becomes established there is a corresponding decrease in the cellular immune response.

Humoral immune response induced by the prepared vaccines were comparatively evaluated for chick groups vaccinated with combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccine using either Montanide™ ISA 71R VG and Paraffin oils as adjuvants.

For AIV H5N1, chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant vaccine, showed increased mean log2 HI antibody titer (5.33 log2) from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (10 log2) at the 3rd WPV and remained in suitable levels (4.33 log2) till 31st WPV. On the other hand, chicks vaccinated with paraffin oil adjuvant vaccine, showed increased mean log2 HI antibody titer (4 log2) from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (8.3 log2) at the 5th WPV, then declined to (4.3 log2) at the 21st WPV then declined gradually to reach the lowest HI antibody titer (1.33 log2) at the 31st WPV (table 5).

For AIV H9N2, chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant vaccine, showed increased mean log2 HI antibody titer (5.6 log2) from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (10 log2) at the 4th WPV and remained in suitable levels (4.33 log2) till 31st WPV. Chicks vaccinated with paraffin oil adjuvant vaccine, showed increased mean log2 HI antibody titer (3 log2) from the 1st week post vaccination (WPV), then reached the highest HI antibody titer (7.3 log2) at the 4th WPV, then declined to (4 log2) at the 23rd WPV then declined gradually to reach the lowest HI antibody titer (1.0 log2) at the 31st WPV (table 6). The results similar to Swayne et al., (1997) and Swayne et al., (2000) who proved that the best vaccines produce protection beginning in 7 to 10 days after vaccination with the peak protection at 3 to 4 weeks and protection may

last up to 6 to 12 months. Also, these results came in accordance with that of Qiau et al., (2006) who proved the maximal level of antibodies of (highly pathogenic strain A/goose/Guangdong/1/96 (H5N1) 1:1024 was recorded on the 6th week. Also, these findings came in agreement with that of Zhailyaubay et al., (2010) who showed that the high immunogenicity of the AI- inactivated emulsified vaccine H5N1 developed by using Montanide™ ISA- 70 (Seppic, France) as oil adjuvant, showing hemagglutinating antibodies were detected in 14 days and reached their peak of 1:277 on the 6th week post vaccination.

Concerning IBV, chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant vaccine, showed increased antibody titer measured by mean ELISA optical density (453) from the 1st WPV, then reached the highest values (2108) at the 4th WPV and remained in suitable levels (1204) till 21st WPV. Chicks vaccinated with paraffin oil adjuvant vaccine, showed increased antibody titer measured by mean ELISA optical density (586) from the 1st WPV, then reached the highest HI antibody titer (1862) at the 4th WPV, then declined to (542) at the 21st WPV (table 7). Finding came in agreement with that of Sultan et al., (2004) and El-Mahdy et al., (2010) who clarify the role of the Mass41 (M41) strain which is commonly used in inactivated vaccines, also the finding came in agreement with that of Muneer et al., (1986) who proved that the inactivated oil-emulsion IBV vaccines are commonly used to obtain long-lasting immunity to protect breeders and layers prior to the onset of the egg production. The results also like Tewfik et al., (2013) who showed D88 strain was the best protectotype where it gave an excellent immune response at the 3rd day post challenge.

Both vaccinated and non-vaccinated control chicks were challenged 28 days post vaccination using virulent strains of AIVs H5N1, H9N2 and IBV. The protection percent against HPAIV H5N1 were 100% in chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant vaccine and 84% in chicks vaccinated with paraffin oil adjuvant vaccine (table 8). These results came in accordance with that of Zhailyaubay et al., (2010) who showing hemagglutinating inhibiting antibodies were detected in 14 days and reached their peak of 1:277 on the 6th week post vaccination. 100% protection against infection since 28 day post vaccination up to 150 days; then slowly going down to 80% (the rate sufficient for ensuring safety of the vaccinated poultry) by the 360th day post vaccination.

Evaluation of shedding of AIV H9N2 were zero % using RRT-PCR in challenged chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant vaccine and paraffin oil adjuvant vaccine, compared with 100% shedding for control non-vaccinated chicks in tracheal swaps at 2nd, 4th and 6th day post challenge as shown in table (9). These results came in accordance with Jeong et al, (2015) who proved that the administration of the inactivated AIV H9N2 vaccine is highly effective in decreasing the clinical signs and virus shedding of flocks. Also, these results disagree with Swayne et al., (1997; Swayne et al., (2000) who proves that the vaccines may protect from morbidity and mortality but not reduce replication and shedding from respiratory and digestive tracts also, the greater the genetic similarity between the HA of vaccine and field viruses, the greater is the reduction in challenge virus replication and shedding from the respiratory tract.

Evaluation of shedding of IBV were zero % using RRT-PCR in challenged chicks vaccinated with Montanide™ ISA 71-RVG oil adjuvant vaccine but the shedding of IBV using RRT-PCR in challenged chicks

vaccinated with paraffin oil adjuvant vaccine, were zero % at 2nd day post challenge then shedding occurred at 4th and gradually declined 6th day post challenge, compared with 100% shedding for control non-vaccinated chicks in tracheal swaps at 2nd, 4th and 6th day post challenge as shown in table (10). These Finding came in agreement with that of Tewfik et al., (2013) who showed D88 strain was the best protectotype where it gave an excellent immune response at the 3rd day post challenge. The protection % was 100% in both trachea and kidney virus re-isolation

In conclusion, combined inactivated AIVs H5N1, H9N2 and IBV oil emulsion vaccine using either Montanide™ ISA 71R VG and Paraffin oils as adjuvants showed suitable cellular and humoral immune responses in vaccinated chicks but with superior results for the vaccine with Montanide™ 71-RVG adjuvant that also provided 100% protection percent for AIV H5N1 and no secretion of AIV H9N2 and IBV in comparison to the combined vaccine with paraffine oil adjuvant that gave 84% protection percent for AIV H5N1 and secretion of AIV H9N2 and IBV occurred 6th day post challenge.

Finally, Montanide™ 71-RVG adjuvant has the flexible ratio of oil and antigenic media in the vaccine (60:40) allowed using large amount of virus that showed good impact on its immunogenicity, other than mineral oil adjuvant which is restricted Ratio of oil and antigenic media of vaccine (73:27).

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