



# Trial for Preparation and Evaluation of Combined Inactivated Vaccine for the Protection against *Riemerella anatipestifer* and Avian Influenza (H5N1) in Ducks

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**Abstract** | *Riemerella anatipestifer* and Avian Influenza infections cause major economic losses in the duck industry through high mortality rates, poor feed conversion, increased condemnations, and high treatment costs. A field trial was conducted to evaluate the immunogenicity of an experimentally multivalent inactivated *R. anatipestifer* and Avian Influenza (H5N1) vaccine. In this study a combined inactivated Montanide ISA 71 *R. anatipestifer* and Avian Influenza (H5N1) vaccine was prepared then the immunization potency and protective efficacy were evaluated through experimental application on Pekin ducklings. The peak of humeral immune response against *R. anatipestifer* as measured by indirect haemagglutination inhibition test (HI) was at the 6<sup>th</sup> week post primary vaccination while it was at the 4<sup>th</sup> week when boosting 3 weeks after primary vaccination and 3<sup>rd</sup> week when boosting 8 weeks after primary vaccination; meanwhile AI immune response as measured by (HI) reached the peak at the 5<sup>th</sup> week post primary vaccination and at 4<sup>th</sup> week for single AI and 5<sup>th</sup> week for combined RA and AI vaccines when boosting 3 weeks after primary vaccination and 5<sup>th</sup> week when boosting 8 weeks after primary vaccination. Regarding the challenge test, the protection percentage were 70% against both RA<sub>1</sub> and RA<sub>2</sub> virulent strains in RA vaccine and 75% in combined RA and AI vaccine after challenge 3 weeks post primary vaccination, while it was 80% against RA<sub>1</sub> and RA<sub>2</sub> virulent strains in RA vaccine and 85% in combined RA and AI vaccine after challenge 3 weeks post boosting. In conclusion the combined prepared *R. anatipestifer* and Avian Influenza (H5N1) vaccine succeeded in eliciting protective antibody titers and satisfactory protection against both *R. anatipestifer* and Avian Influenza (H5N1) diseases.

**Keywords** | *R. anatipestifer*, AI, vaccine; HI, Challenge test

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## INTRODUCTION

*Riemerella anatipestifer* and Avian Influenza cause major economic losses in duck industry through high mortality rates, poor feed conversion, increased condemnations, and high treatment costs (Kardos et al., 2007; Sun et al., 2012). *R. anatipestifer* infection causes mortality rates between 10 and 75% in ducks fewer than 8 weeks of age in the infected farms (Subramaniam et al., 2000). Now a day,

21 serotypes have been identified in the world, and there is poor cross-protection among these serotypes (Kardos et al., 2007). Infections from *R. anatipestifer* serotypes 1, 2, 3, 5, 6, 7, 8, 10, 11, 13, 14, and 15 have been reported in China; however, serotypes 1, 2 are responsible for most of the major outbreaks (Hu et al., 2001). Signs usually include head shaking, lethargy and an abnormal gait. Post mortem signs in acute cases include enlargement of the liver and spleen and lung congestion. In more chronic cases, pericar-

ditis, perihepatitis and air sacculitis with a caseous deposit can be found (Sandhu, 1986; Higgins et al., 2000).

Various antibiotics are currently used to prevent and control *R. anatipestifer* infection in ducks, but they accelerate the emergence of drug-resistant strains (Chen et al., 2010; Chen et al., 2012). The resistance of *Pasteurella multocida* and *R. anatipestifer* for many antibiotics has increased greatly, and antibiotic residues have been detected in duck-related products (Sun et al., 2012). So, vaccine application is the most accepted method for the control of the diseases, by obtaining a high level of antibodies, rather than using other eradication or medication strategies. Vaccines based on a single serotype of inactivated bacterin have not provided significant cross-protection among the serotypes (Sandhu, 1979).

Highly pathogenic avian influenza virus (HPAIV) H5N1 (A/H5N1) caused enormous economic losses in poultry in many countries worldwide and genetically diversified into 10 clades and several subclades since 1996/1997 (Smith and Donis, 2015). Egyptian A/H5N1 of clade 2.2.1 has diversified into several genetic groups. Most of these phylogroups disappeared but two major clades circulated for several years (Naguib et al., 2016a). Clade 2.2.1.1 represented antigenic-drift variants, which were primarily isolated from vaccinated commercial poultry leading to three human infections so far according to the official reports to the (WHO, 2017). These viruses appeared in early 2007 and predominated in 2008–2010 challenging the efficacy of the highly diverse H5 vaccines in Egypt. In 2011–2014, the prevalence of 2.2.1.1 viruses dramatically decreased and they are most likely extinct by now (Naguib et al., 2016b; El-Shesheny et al., 2017; Rohaim et al., 2017). The second clade is 2.2.1.2 viruses which circulated in non-vaccinated backyard birds and were introduced into small-scale farmed poultry since early 2008. The vast majority of infected humans were infected by this genotype (Younan et al., 2013). In 2014/2015, an unprecedented upsurge of 2.2.1.2 was reported in poultry and humans marking Egypt as the country with the highest number of human infections with A/H5N1 worldwide (Arafa et al., 2015; WHO, 2017). These viruses spread to neighboring countries posing a serious threat in the Middle East (Naguib et al., 2016a; Salaheldin et al., 2017). Driving forces for the emergence, extinction or spread of A/H5N1 clades in Egypt are not well-studied except for the massive application of vaccines and antivirals in poultry (Abdel-Moneim et al., 2011; Cattoli et al., 2011). Improved vaccination strategy is required to increase the immunogenicity in chicken farms. These strategies could include: the use of a highly immunogenic vaccine seed strain, the improvement of an effective adjuvant for chickens, and the consideration of a 2-dose regimen (Dong-Hun Lee et al., 2013). To reach optimal protec-

tion, the vaccine should be matching to the field strain and reduced shedding of the virus. As the antigenic drift of AI viruses after extended vaccination programs has been observed in chickens, similar to what has been observed with human influenza viruses (Suarez et al., 2006). Using a recently developed plasmid-based reverse genetics system for influenza, viruses can be rescued from cloned cDNA that enables the rapid generation of reassortant influenza virus, using a plasmid-only rescue system, (Hoffmann et al., 2002) described the usefulness of the reverse genetics system for the rapid generation of reassortants for human influenza vaccine. In addition, it can convert High pathogenic avian influenza (HPAI) into Low pathogenic avian influenza (LPAI) vaccine candidates by mutating the HA cleavage site, allowing an easier and safer handling for vaccine manufactures (Subbarao and Kafz, 2004). Two AI-inactivated vaccines (a homologous H5N1 and a heterologous H5N3) using this technology have recently been licensed (Liu et al., 2003). These vaccines which contain a homologous HA gene have been shown to be more protective in ducks and geese than conventional vaccines with a more distant HA gene (Middleton et al., 2007).

Because of both Avian influenza and *R. anatipestifer* infections cause economic losses, occur at about the same age, and have same signs and post-mortem lesions, the aim of work is the development of a combined inactivated vaccine against *R. anatipestifer* and AI as a convenient product for breeding stock, where it can be injected in one shot for the purposes of covering the above mentioned points, it is suggested that such vaccine could be able to protect ducks against field challenges exhibiting them good immunity levels which can be passed to the next generation through the egg yolk.

## MATERIALS AND METHODS

### STRAINS USED IN VACCINE PREPARATION

**Riemerella anatipestifer:** *Riemerella anatipestifer* the local isolates of RA<sub>1</sub> and RA<sub>2</sub> were obtained from Aerobic bacterial vaccines department in VSVRI/ARC Egypt and previously identified morphologically, biochemically and genetically.

**Avian Influenza H<sub>5</sub>N<sub>1</sub>:** For H5N1 vaccine strain preparation (RGD10552B/H5N1), a plasmid-based reverse genetics system was applied using 6 internal genes of A/Puerto Rico/8/34(H1N1) and 2 surface LP-HA and NA genes of D10552B/H5N1 virus. Field isolate attenuated by reverse genetics and provided from National Research Center/ Egypt. (Mokhtar et al., 2019).

### ADJUVANT

Montanide™ ISA 71 VG. It is a mineral oil based adju-

vant that has been developed for manufacture of water-in-oil (W/O) emulsion. The adjuvant was kindly provided by SEPPIC Co, France.

### VACCINE PREPARATION

**Preparation of inactivated *Riemerella anatipestifer* antigenic phase:** It was according to (Pathanasophon et al., 1996): *R. anatipestifer* isolates were cultured separately in Tryptic Soya Broth (TSB) at 37°C for 24 hours with shaking. The count of bacterial CFU for each strain was adjusted to  $1.4 \times 10^{10}$  CFU/ml for each one. The bacteria were then inactivated with 0.5 % formalin at 37°C for 24 hours. After inactivation each culture was tested for purity, safety and sterility as mentioned by (OIE, 2019). Finally, cultures were equally mixed together then preserved with 0.01% of thiomersal and stored at 4°C until used.

**Preparation of inactivated AIH<sub>5</sub>N<sub>1</sub> antigenic phase:** Propagation and titration of AI H<sub>5</sub>N<sub>1</sub> were done in SPF eggs 9 -10 day old Embryonated Chicken Egg (ECE) according to (OIE, 2019). Its titer was  $10^9$  EID<sub>50</sub>/ml. Inactivation of AI virus was carried out using formalin with final concentration 0.01% of total volume at 25°C for 18 hrs. After inactivation sodium bisulfite was added as a final concentration of 2% to stop the action of formalin and stored at 4°C until used. The antigen content in all the prepared vaccines must be not less than  $10^{8.5}$  embryo infective dose 50% (EID<sub>50</sub>). Inactivation and testing of inactivation was done according to (OIE, 2019).

**Formulation of inactivated *Riemerella anatipestifer*, inactivated Avian influenza and combined inactivated *Riemerella anatipestifer* and AI vaccines:** The inactivated *Riemerella anatipestifer*, the inactivated avian influenza and the combined inactivated *Riemerella anatipestifer* and AI vaccines were prepared by the formulation process recommended by Montanide manufacture. W/O emulsion vaccine using Montanide™ ISA 71 VG at a ratio of 30/70 (W/W) aqueous/oil ratio was prepared. The same for combined inactivated *R. anatipestifer* and AI vaccines but we added equal weight for both AI & *R. anatipestifer* taking in consideration that the antigenic content for both antigens not less than  $10^8$  EID<sub>50</sub>. Stable preparations were obtained by mixing the aqueous medium into the Montanide™ ISA 71 VG, at room temperature or less, under vigorous stirring (for 15-30 min) as recommended by manufacture.

### QUALITY CONTROL OF THE PREPARED VACCINE

The prepared vaccines were tested for sterility, safety and purity tests following the standard international protocols as described by (CFR, 2019).

### EXPERIMENTAL DUCKS

Two hundred and fifty, one day-old apparently healthy Pe-

kin ducklings obtained from commercial farm at El-Minufiya Governorate were used in this study. The ducks were kept and reared in separate pens at the animal husbandry facilities of Central Laboratory for Evaluation Veterinary Biologics (CLEVB) until used for the experiment. Blood samples were collected directly before the experiment and serological examinations (IHA, HI) were done to ensure that the ducks have no maternal or acquired antibodies against *R. anatipestifer* or AI.

### EXPERIMENTAL DESIGN

A total of 250 ducklings four weeks old were divided into four groups, the 1st group (90 ducks) was vaccinated with single dose of the prepared inactivated oil emulsion *R. anatipestifer* vaccine. The 2nd group (20 ducks) was vaccinated with single dose of the prepared inactivated oil emulsion AI vaccine. The 3rd group (90 ducks) was vaccinated with single dose of the prepared inactivated oil emulsion combined *R. anatipestifer* and AI vaccine. The 4th group (50 ducks) was remained as control unvaccinated group.

The 1st and 3rd groups were divided into 3 subgroups and inoculated as follow:

Subgroup (a) (40 ducks): received single dose then challenged 3 weeks post vaccination. Subgroup (b) (40 ducks): received first dose and booster dose 3 weeks post vaccination then challenged 3 weeks post boosting. Subgroup (c) (10 ducks): received single dose and the titer was followed up until decreased then received booster dose.

The 2<sup>nd</sup> group was divided into two equal subgroups, subgroup (a) (10 ducks): received first dose and booster dose 3 weeks post vaccination and subgroup (b) (10 ducks) received single dose and the titer was followed up until decreased then received booster dose. Table (1).

### VACCINE INOCULATION

The ducks in each group were inoculated S/C with 0.5ml of vaccines at the dorsal aspect of the neck.

### BLOOD SAMPLES COLLECTION

Random blood samples were collected weekly post immunization to evaluate the developed immune response to the vaccines.

### SEROLOGICAL TESTS FOR EVALUATION OF HUMERAL IMMUNE RESPONSE OF PREPARED VACCINES

**Indirect haemagglutination test (IHA):** IHA test was used for evaluation of humeral immune response against *R. anatipestifer*. The glutaraldehyde RBCs and capsular antigen of *R. anatipestifer* were prepared according to (Carter and Cole, 1990). A vaccine is considered efficient as induce sera conversion in the sera of vaccinated ducks.

**Table 1:** The experimental design

Groups	1			2		3		
Vaccines	Inactivated RA vaccine			Inactivated AI vaccine		Combined inactivated RA and AI vaccine		
Subgroups	c	b	a	b	a	c	b	a
	Received single dose and the antibody titer was followed till decreased then received booster dose	Received first dose then booster dose 3 weeks after vaccination then challenged 3 weeks after boosting	Received single dose then challenged after vaccination	Received single dose and the antibody titer was followed till decreased then received booster dose	Received first dose then booster dose 3 weeks after vaccination then challenged 3 weeks after boosting	Received single dose and the antibody titer was followed till decreased then received booster dose	Received first dose then booster dose 3 weeks after vaccination then challenged 3 weeks after boosting	Received single dose then challenged after vaccination

RA: *Riemerella anatipestifer*

AI: Avian influenza

**Table 2:** IHA antibody titers in sera of ducklings vaccinated with single dose of inactivated *R. anatipestifer* and combined *R. anatipestifer* and AI vaccines

Weeks post primary vaccination	Type of vaccines				Control
	Inactivated RA vaccine		Combined inactivated RA and AI vaccine		
	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	
Pre-vaccination	2	2	2	2	0.0
2 <sup>nd</sup> week	16	8	16	16	0.0
3 <sup>rd</sup> week	16	16	32	32	0.0
4 <sup>th</sup> week	32	32	64	64	0.0
5 <sup>th</sup> week	128	64	256	128	0.0
6 <sup>th</sup> week	512	256	1024	512	0.0
7 <sup>th</sup> week	256	128	512	256	0.0
8 <sup>th</sup> week	128	64	128	128	0.0

RA: *Riemerella anatipestifer*

AI: Avian Influenza

**Heamagglutination Inhibition test (HI):** It was used for evaluation of humeral immune response against AI. It was performed according to (OIE, 2019).

**CHALLENGE TEST**

The vaccinated and control groups of ducks were challenged with 0.1 of 10<sup>8</sup>CFU of each *R. anatipestifer* strains RA1 and RA2 intramuscularly (Babars, 2000). Birds after challenge were observed daily for a week for any mortality or clinical signs. The clinical findings of both the vaccinated and unvaccinated birds were observed and recorded.

**STATISTICAL ANALYSIS**

P Values of outcome data were calculated and analyzed using SPSS program version 21 (2012).

**RESULTS**

**RESULTS OF QUALITY CONTROL OF PREPARED VACCINES**

**Purity test:** The Culture of *R. anatipestifer* and AI virus used in prepared vaccines were confirmed to be pure.

**Sterility test:** The prepared vaccines were free from bacterial, fungal and Mycoplasma contamination according to (OIE, 2019).

**Safety test:** The vaccines were found to be safe and produced no clinical, local, systemic or necropsy findings during two weeks observation period of the injected ducks.

**INDIRECT HAEMAGGLUTINATION TEST (IHA) AGAINST *R. ANATIPESTIFER* STRAINS IN VACCINATED DUCKS**

As shown in Table (2), the serological test carried out on serum samples obtained from ducks vaccinated with sin



**Table 3:** IHA antibody titers in sera of ducklings received booster dose 3 weeks and 8 weeks after primary vaccination of inactivated *R.anatipestifer* and combined *R. anatipestifer* and AI vaccines.

Weeks post boosting	Boostering 3 weeks post primary vaccination				Boostering 8 weeks post primary vaccination after the titer decreased				Control
	Inactivated RA vaccine		Combined inactivated RA and AI vaccine		Inactivated RA vaccine		Combined inactivated RA and AI vaccine		
	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	
1 <sup>st</sup> week PB	64	32	128	64	256	128	256	256	0.0
2 <sup>nd</sup> week PB	128	64	256	128	512	256	512	512	0.0
3 <sup>rd</sup> week PB	256	128	512	256	1024	512	1024	1024	0.0
4 <sup>th</sup> week PB	512	256	1024	512	1024	512	1024	1024	0.0
5 <sup>th</sup> week PB	512	256	1024	512	512	256	1024	1024	0.0
6 <sup>th</sup> week PB	256	128	512	256	256	128	512	512	0.0
7 <sup>th</sup> week PB	128	64	256	128	128	64	256	128	0.0

RA: *Riemerella anatipestifer*      AI: Avian Influenza      PB: Post boosting

**Table 4:** HI log<sub>2</sub> titer in sera of ducklings vaccinated with single dose of inactivated AI and combined *R. anatipestifer* and AI vaccines.

	Type of vaccines		Control
	Inactivated AI vaccine	Combined inactivated RA and AI vaccine	
Pre-vaccination	0	0	0
2 <sup>nd</sup> week	6	6.6	0
3 <sup>rd</sup> week	6.8	7.6	0
4 <sup>th</sup> week	7.4	8.2	0
5 <sup>th</sup> week	8	9	0
6 <sup>th</sup> week	8	8.6	0
7 <sup>th</sup> week	6.2	7.8	0
8 <sup>th</sup> week	5.8	6.4	0

RA: *Riemerella anatipestifer*      AI: Avian Influenza

gle dose of inactivated RA and combined RA and AI vaccines revealed that such birds exhibited detectable *R. anatipestifer* antibodies from the second week post vaccination. The antibody titer increased gradually till reached its peak at 6<sup>th</sup> week post vaccination where it reached 512 and 256 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the *R. anatipestifer* vaccine while 1024 and 512 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the combined inactivated *R. anatipestifer* and AI vaccine. The antibodies titer was gradually decreased till reached 128 and 64 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the *R. anatipestifer* vaccine while 128 for both RA<sub>1</sub> and RA<sub>2</sub> strains in the combined *R. anatipestifer* and AI vaccine at the 8<sup>th</sup> week post vaccination. Also from this table, the combined RA and AI vaccine showed higher level of antibody titers when compared with the *R. anatipestifer* vaccine and the antibody titers were decreased at 8<sup>th</sup> week so this subgroups (c) were received a booster dose of vaccines at 8<sup>th</sup> week. The p values for R1 and R2 strains between mono and combined vaccines were 0.110 and 0.05 respectively.

As shown in Table (3), the inoculation of a booster dose 3 weeks post primary vaccination (subgroup b) caused rapid rise of antibody titers till reached the peak at 4<sup>th</sup> week post boosting where it reached 512 and 256 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the RA vaccine while 1024 and 512 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the combined RA and AI vaccine that persist for the 5<sup>th</sup> week then gradually decreased till reached 128, 64 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the RA vaccine while 256 and 128 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the combined RA and AI vaccine at 7<sup>th</sup> week post boosting. On the other hand, the inoculation of a booster dose 8 weeks post primary vaccination after the titer was decreased (subgroup c) caused increase in the titer till reached the peak at 3<sup>rd</sup> week post boosting where it reached 1024 and 512 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in the RA vaccine while 1024 in the combined RA and AI vaccine that persist for 4<sup>th</sup> week in RA vaccine and persist for 5<sup>th</sup> week in the combined vaccine then gradually decreased till reached 128, 64 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in RA vaccine and 256

**Table 5:** HI log<sub>2</sub> titer in sera of ducklings received booster dose 3wks and 8wks after primary vaccination of inactivated AI and combined *R. anatipestifer* and AI vaccines

Weeks post boosting	Boostering 3 weeks post primary vaccination		Boostering 8 weeks post primary vaccination after the titer decreased		Control
	Inactivated AI vaccine	Combined RA and AI vaccine	Inactivated AI vaccine	Combined RA and AI vaccine	
1 <sup>st</sup> week PB	7.6	8	6.8	7.4	0
2 <sup>nd</sup> week PB	8	8.4	7.7	8.2	0
3 <sup>rd</sup> week PB	8.2	8.6	8.2	8.6	0
4 <sup>th</sup> week PB	8.4	8.7	8.4	8.8	0
5 <sup>th</sup> week PB	8.4	9	8.6	9	0
6 <sup>th</sup> week PB	8	8.4	8.4	8.7	0
7 <sup>th</sup> week PB	7.2	7.6	8	8.4	0

RA: *Riemerella anatipestifer* AI: Avian Influenza PB: Post boosting

**Table 6:** Mean protective efficacy in ducks vaccinated with RA and combined RA and AI vaccines that challenged 3 weeks after primary vaccination and 3 weeks post boosting.

	Challenge 3weeks post vaccination (subgroup a)						Challenge 3 weeks post boosting (subgroup b)					
	RA vaccine		RA+AI		Control		RA vaccine		RA+AI		Control	
	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>	RA <sub>1</sub>	RA <sub>2</sub>
No. of challenged ducks	20	20	20	20	5	5	20	20	20	20	5	5
No. of died ducks	6	6	5	5	4	4	4	4	3	3	4	4
Protection %	70	70	75	75	20	20	80	80	85	85	20	20

RA: *Riemerella anatipestifer* AI: Avian Influenza

Subgroup (a): ducks that received one dose of vaccine then challenged after 3 weeks.

Subgroup (b): ducks that received booster dose of vaccine 3 weeks post vaccination then challenged after 3 weeks post boosting.

and 128 for RA<sub>1</sub> and RA<sub>2</sub> strains respectively in combined RA and AI vaccines at 7<sup>th</sup> week post boosting.

Also from this table, the combined RA and AI vaccine showed higher level of antibody titers after inoculation of booster dose when compared with the *R. anatipestifer* vaccine.

The p values for R1 and R2 strains after 3 weeks boosting between mono and combined vaccines were 0.009 for the two strains while they were 0.134 and 0.007 for 8 weeks boosting respectively.

### HAEMAGGLUTINATION INHIBITION TEST AGAINST AVIAN INFLUENZA IN VACCINATED DUCKS

As shown in Table (4), the serological test carried out on serum samples obtained from ducks vaccinated with single dose of inactivated AI and combined RA and AI vaccines revealed that such birds exhibited detectable AI antibodies from the second week post vaccination. The antibody titer increased gradually till reached its peak at 5<sup>th</sup> week post vaccination where it reached 8 HI log<sub>2</sub> in AI vaccine and 9 HI log<sub>2</sub> in combined RA and AI vaccine that persist for

6<sup>th</sup> week. The antibodies titer was gradually decreased till reached 5.8 and 6.4 in AI vaccine and combined RA and AI vaccine respectively at 8<sup>th</sup> week post vaccination. Also from this table, the combined RA and AI vaccine showed higher level of antibody titers when compared with the AI vaccine and the antibody titers were decreased at 8<sup>th</sup> week so this subgroup were received a booster dose of vaccines at 8<sup>th</sup> week. The p values between mono and combined vaccines were 0.001.

From Table (5), the inoculation of a booster dose 3 weeks post primary vaccination caused rapid rise of antibody titers till reached the peak 8.4 HI log<sub>2</sub> in AI vaccine at 4<sup>th</sup> week post boosting while it reached its peak 9 HI log<sub>2</sub> at 5<sup>th</sup> week post boosting in combined RA and AI vaccine. The antibody titers persisted for another week then decreased till reached 7.2 in AI vaccine and 7.6 in combined RA and AI vaccine at 7<sup>th</sup> week post boosting. On the other hand, the inoculation of a booster dose 8 weeks post primary vaccination after the titer was decreased caused increase in the antibody titer till reached the peak 8.6 HI log<sub>2</sub> in AI vaccine while it reached its peak 9 HI log<sub>2</sub> in combined RA and AI vaccine at 5<sup>th</sup> week post boosting.

The antibody titers decreased till reached 8 in AI vaccine and 8.4 in combined RA and AI vaccine at 7<sup>th</sup> week post boosting.

The p values between mono and combined vaccines when boosting at 3 weeks and when boosting at 8 weeks were 0.000.

### RESULTS OF CHALLENGE TEST

As shown in Table (6), evaluation of the protection rate in the vaccinated ducks using challenge test by *R. anatipestifer* strains (RA<sub>1</sub> and RA<sub>2</sub>) in the (subgroup a) which received single dose then challenged after 3 weeks cleared that in inactivated RA vaccine, the protection percent was 70% for both RA<sub>1</sub> and RA<sub>2</sub> strains. Meanwhile in combined inactivated RA and AI vaccine, the protection percent was 75% for both RA<sub>1</sub> and RA<sub>2</sub> strains. On the other hand, the protection percent by inoculation of booster dose (subgroup b) was 80% for both RA<sub>1</sub> and RA<sub>2</sub> strains in RA vaccine while it was 85% for RA<sub>1</sub> and RA<sub>2</sub> strains in combined RA and AI vaccine 3 weeks post boosting. The protection percent in unvaccinated control group was 20% in both vaccines.

### DISCUSSION

*Riemerella anatipestifer* infection (RA) causes major economic losses in duck, through high mortality rates, poor feed conversion, increased condemnations, and high treatment costs (Kardos et al., 2007; Sun et al., 2012).

*R. anatipestifer* causes disease in ducks throughout the world. Formerly known as *pasteurella anatipestifer*, that usually causes disease in young ducklings aged between 2 and 6 weeks. Stress factors such as moving birds and environmental variations can trigger disease. Signs usually include head shaking, lethargy and an abnormal gait. Post mortem signs in acute cases include enlargement of the liver and spleen and lung congestion. In more chronic cases, pericarditis, perihepatitis and air sacculitis with a caseous deposit can be found (Sandhu, 1986; Higgins et al., 2000).

Control of *R. anatipestifer* and avian influenza is still subject of interest and usually attract attention of researchers to know more about diseases, epidemiology and how to control in susceptible birds. Combination of bacterial and viral vaccines has many benefits for the manufacturer as it reduces production costs, save time, effort and simplifies the immunization schedule. Also it minimizes stress of multiple vaccinations on the animals. Vaccination is still considered one of the major tools for controlling both of the two diseases. Vaccine efficacy depends on many variables, such as the nature, the amount of antigen adminis-

trated and the presence of adjuvant to enhance immunogenicity. New resisting adjuvants more and more needed to ensure proper stability profiles of vaccines so, in this study three vaccines were produced, inactivated RA, inactivated AI and combined inactivated RA and AI vaccines using a flexible water in oil emulsion Montanide 71VG adjuvant able to resist destabilizing antigenic media without impairing the safety and efficacy of avian vaccine (Arous et al., 2013)

Regarding the potency of the prepared vaccine, the serological tests carried out on serum samples obtained from vaccinated ducks with the combined RA and AI vaccine group (3) revealed that such birds exhibited detectable *R. anatipestifer* antibodies from the second week post vaccination. Evaluation of the antibody titer in sera of the vaccinated ducklings using the IHA test which received single dose, cleared that the superiority of the inactivated combined vaccine than the monovalent vaccine, where the IHA antibody titer increased gradually till reach its maximum level 512, 256, 1024 and 512 in monovalent (RA<sub>1</sub>), (RA<sub>2</sub>) group (1) and combined vaccine (RA<sub>1</sub>), (RA<sub>2</sub>) group (3) respectively at 6<sup>th</sup> week post first vaccination then gradually decreased till reached 128, 64, 128 and 128 in monovalent (RA<sub>1</sub>), (RA<sub>2</sub>) group (1) and combined vaccine (RA<sub>1</sub>), (RA<sub>2</sub>) group (3), respectively at the 8<sup>th</sup> week post first vaccination. Also it was noted that combined vaccine showed higher level when compared with the results of ducklings vaccinated with monovalent *R. anatipestifer*. The obtained results agreed with that of, (Amina et al., 2003; AboulSaoud, 2010; Pathansophon et al., 1996) who reported that antibody titers were higher at 5, 6 weeks post 1<sup>st</sup> vaccination.

The inoculation of booster dose 3 weeks apart which caused rapid rise of antibodies titer till reached the peak 512, 256, 1024 and 512 in monovalent (RA<sub>1</sub>), (RA<sub>2</sub>) group (1) and combined vaccine (RA<sub>1</sub>), (RA<sub>2</sub>) group (3), respectively at 4<sup>th</sup> week after booster dose that persist for 5<sup>th</sup> week then gradually decreased till reached 128, 64, 256 and 128 in monovalent (RA<sub>1</sub>), (RA<sub>2</sub>) group (1), and combined vaccine (RA<sub>1</sub>), (RA<sub>2</sub>) group (3), respectively at 7<sup>th</sup> week PB. The obtained results agreed with (Eman et al., 2020). Who noted that all birds showed a secondary response greater than that seen in birds vaccinated once. These findings were in accordance with that recorded by (Jabbri et al., 2005) who stated that a significant immunological stimulus had been elicited by the 2<sup>nd</sup> exposure and (Iqbal et al., 2010) who reported that the immune response of secondary immunization was significantly higher than the primary vaccination.

Inoculation of 2<sup>nd</sup> dose after decreasing of antibody titers at the 8<sup>th</sup> week post primary vaccination stated that antibody titers in the sera of ducklings increased gradually

till reached the maximum at 3<sup>th</sup> week (1024, 512, 1024 and 1024) that persist for 4<sup>th</sup> week in RA vaccine and for 5<sup>th</sup> week in combined RA and AI vaccine then decreased gradually till reached 128, 64, 256 and 128 at 7<sup>th</sup> week.

Avian influenza (AI) is a major zoonotic viral disease that causes significant adverse impacts on poultry production, the global trade, and public health (Capua et al., 2002; Alexander DJ, 2007). Despite decades of research and control efforts, the incidences and severity of AI outbreaks have not been alleviated but rather increased (CDC, 2017; USDA, 2017).

The most important part of immune response for controlling of AIV is the humeral immunity (Wareing and Tannock, 2001).

The obtained results revealed that HI titres of birds with single shot of bivalent H5N1 (AI) and *Riemerella* vaccine group (3) compared to inactivated H5N1 (AI) vaccine group (1) showed gradual increase in the antibody titer where, the maximum HI titer of birds was 9 & 8 log<sub>2</sub> HI titer respectively in 5<sup>th</sup> week post primary vaccination that persist for 6<sup>th</sup> week then gradually decreased till reached 6.4 and 5.8 in combined and single vaccines respectively. These results agreed with (Zhao et al., 2017) who reported that the vaccine could induce a fast immunological reaction and the mean HI titers were over 6.9 log<sub>2</sub> against both NDV and H9N2 AIV at 2 wpv. At 4 wpv, peak levels of HI antibodies were detected with a mean HI titer of 8.6 log<sub>2</sub> against NDV and 9.5 log<sub>2</sub> against H9N2.

The results of birds boosted with prepared bivalent H5N1 (AI) and *Riemerella* vaccine compared to inactivated H5N1 (AI) group (2) vaccine showed the maximum titers 9, 8.4 log<sub>2</sub> respectively at 5<sup>th</sup> week post boosting 3 weeks after primary vaccination while it was 9 and 8.6 log<sub>2</sub> post boosting 8 weeks after primary vaccination and decreased gradually till the 7<sup>th</sup> week. Regarding the statistical analysis, there is a significant difference between inactivated RA vaccine and inactivated combined RA and AI vaccines, also between inactivated AI vaccine and combined inactivated RA and AI vaccine, these results agree with (Mokhtar et al., 2019) who found that the use of polyvalent vaccines lead to better vaccination coverage.

Regarding the challenge test, the evaluation of the protection rate in the vaccinated ducks using challenge test by *R. anatipestifer* strains (RA<sub>1</sub> and RA<sub>2</sub>) in the (subgroup a) which received single dose then challenged after 3 weeks cleared that, in inactivated RA vaccine, the protection percent was 70% for both RA<sub>1</sub> and RA<sub>2</sub> strains. Meanwhile in combined inactivated RA and AI vaccine, the protection percent was 75% for both RA<sub>1</sub> and RA<sub>2</sub> strains. On the other hand, the protection percent by inoculation of

booster dose (subgroup b) was 80% for both RA<sub>1</sub> and RA<sub>2</sub> strains in RA vaccine while it was 85% for RA<sub>1</sub> and RA<sub>2</sub> strains in combined RA and AI vaccine 3 weeks post boosting. The protection percent in unvaccinated control group was 20% in both vaccines.

From these data it was clear that the antibody titer measured with IHA test was highly correlated with protection against challenge with virulent organisms as reported by (Eman et al., 2020). They also stated that the combination increase the antibody titer and protection percent that confirm the obtained results that the protection of ducks vaccinated with combined RA and AI vaccine was higher than RA vaccine and the protection of ducks that received booster dose was higher than that received single dose.

From mentioned data, we have shown that the use of Montanide ISA 71 VG adjuvant induced high and protective long lasting immunity and this finding was confirmed by (Arous et al., 2013) who obtained high and protective antibodies against Newcastle disease antigen and stated that this new adjuvant has been tested successfully in other vaccines for its ability to resist destabilization conditions, and its safety and efficacy performance have been confirmed and can also be adapted to other veterinary vaccines and species.

In conclusion, single shot of oil based vaccine even it confer protective immune response but boost shot of vaccine conferred the maximum protective immune response for longer duration. Polyvalent vaccine strategies increase reactivity for many pathogens including, but not limited to, influenza although polyvalent vaccine formulations clearly expand the breadth of a single vaccine formulation. The reactivity is still limited to the individual components. The goal of polyvalence is to increase the breadth of vaccine coverage by combining diverse components into a single vaccination. So, the combined vaccine was recommended to used not only to reduce the cost of production of two single vaccines, decrease the stress of two injections, or the two diseases affect the same age and have same signs but also for the significant higher immune response of combined vaccine against both disease to protect both duck breeder and duckling from both pathogens.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for



## AUTHORS CONTRIBUTION

All authors contributed equally to study design, sampling, methodology, interpretation of results, and writing of the manuscript.

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