



Preparation of a Combined Inactivated Vaccine against *Riemerella anatipestifer* and Duck Viral Hepatitis

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ABSTRACT

Riemerella anatipestifer (*R. anatipestifer*) infection and duck virus hepatitis (DVH) are enormous dangers for the duck industry and its investment. So, in the current study, a combined inactivated vaccine against both of them was prepared to combat their adverse effect. One hundred and thirty-three ducks of one-day-old of age were used and grouped randomly into four groups. Group (1) was vaccinated with *R. anatipestifer* vaccine, group (2) was vaccinated with DHV vaccine, group (3) was vaccinated with the prepared combined vaccine of both and finally, group (4) was kept as a negative control. Vaccination was at one day old of age. The vaccinated groups with *Riemerella* vaccine had 72.7% protection against challenges with the virulent strain with the highest antibody titers in 6th week as measured by the indirect Hemagglutination test. The control group had 90.9% mortality when challenged against *R. anatipestifer*, with no detectable antibody titers. DVH-vaccinated groups exhibited their highest serum-neutralizing antibody titers by the 5th and 6th weeks post-vaccination. The Control group had no detectable antibody titers against DVH. Statistically, it was clear that there were no significant differences between the results of different groups vaccinated with combined or single vaccines of the same agent. Briefly, combined vaccines of *R. Anatipestifer* and duck viral hepatitis have harmonized effects with the priority to decrease the stress on birds and workers. Besides its efficiency, the economic side as providing one-shot vaccines instead of each one separately.

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INTRODUCTION

Riemerella anatipestifer (*R. anatipestifer*) is one of the most pathogenic bacteria for avian species. *R. anatipestifer* has an adverse effect on the economy in the poultry production sector worldwide. The disease is known as "new duck disease," also variously named infectious serositis, anatipestifer syndrome and duck septicemia. One to 7 weeks is the susceptible age of ducks developing nasal and ocular discharges, head and neck tremors, incoordination, coughing and sneezing. Affected ducklings suffer from lying on their back and paddling movement of legs (typical signs). Necrotic dermatitis may be observed on the lower back or around the vent (Soman *et al.*, 2014). Mortality rates resulting from *R. anatipestifer* infection vary between 10 and 75% in ducklings younger than 8 weeks old (Subramaniam *et al.*, 2000).

Commonly, gross post-mortem lesions are air sacculitis, pericarditis, perihepatitis and fibrinous polyserositis. *R. anatipestifer* may affect the central nervous system causing fibrinous meningitis. Mucopurulent or caseous salpingitis may develop in chronic cases, leading to egg production loss (Soman *et al.*, 2014). Prevention and control of Riemerellosis using various antibiotics accelerate the emergence of drug-resistant strains (Chen *et al.*, 2012). So, the preferred way is the application of a successful vaccine giving high titer of effective antibodies for disease control (Gamal *et al.*, 2021).

Duck hepatitis virus (DHV) is the causative agent of duck viral hepatitis. That disease is defined as an acute and fatal disease of young ducklings. Three serotypes of DHV (DHV-1-3) have been described. DHV-1 is the most widely distributed one and can

cause 90% mortality in ducklings under 3 weeks of age (Ding and Zhang, 2007). It is characterized by liver enlargement, necrosis and hemorrhage (Wang *et al.*, 2022). Adult birds become asymptomatic carriers and shed the virus for a lifetime (Kozdru *et al.*, 2014). Although the disease can be controlled by vaccination of one-day-old ducklings or breeder ducks, the immune response in ducklings is not induced until 3–5 days after vaccination, so vaccination of breeder ducks can protect ducklings from the infection during the interim (Wang *et al.*, 2022).

This study aimed to prepare a combined inactivated vaccine against *R. anatipestifer* and duck viral hepatitis diseases and evaluate the efficacy of single-dose immunization to determine the vaccine efficacy to cover the susceptible age protecting the ducklings simultaneously against both diseases to avoid the stress of repeated injections of young birds with different vaccines.

MATERIALS AND METHODS

Ethical and study protocol approval

This experiment was approved by the Research Committee of the Veterinary Serum and Vaccine Research Institute, Abasia, Agricultural Research Center (VSVRI/ARC), Cairo, Egypt.

Strains used in the vaccine's preparations

Virulent local isolates *R. anatipestifer* 1 and 2 (RA1 and RA2) were obtained from the Aerobic Bacteria Research Department, VSVRI, ARC, Egypt. Vaccinal embryonated chicken egg (ECE) adapted DVH1 (for vaccine preparation) and VERO cell adapted DVH1 (for serum neutralization test) were kindly obtained from Newcastle Disease Research Department, VSVRI, ARC, Egypt.

Adjuvant

Montanide™ ISA 70 VG (SEPPIC Co, France) is a mineral oil-based adjuvant that was used in the vaccine preparation.

R. anatipestifer vaccine preparation

Preparation of inactivated *R. anatipestifer* antigenic phase was done according to Gamal *et al.*, (2021). *R. anatipestifer* isolates were cultured in Tryptic Soya Broth (TSB) at 37° C for 24 hours with shaking. The count of bacterial CFU for each strain was adjusted to 1.4×10^{10} CFU/ml. The bacteria were then inactivated with 0.5 % formalin at 37° C for 24 hours. Then was treated with 20 % sodium bisulfite to make a final concentration of 2% sodium bisulfite, at which the action of formalin was stopped.

Preparation of inactivated DVH antigenic phase

This antigenic fluid was prepared by inoculating vaccinal DVH in 10-day-old ECE. Then it was inactivated by formalin at a final concentration of 0.2 % at room temperature for 24 hours. Then was treated with 20 % sodium bisulfite to make a final concentration of 2% sodium bisulfite at which the action of formalin was stopped. This phase was carried out according to EL-Koffy (1997).

Formulation of inactivated *R. anatipestifer* inactivated DVH and combined inactivated *R. anatipestifer* and DVH vaccines

The inactivated *R. anatipestifer* single (not the combined vaccine), the inactivated DVH (DS) and the combined inactivated *R. anatipestifer* and DVH vaccines were prepared by the formulation process recommended by Montanide manufacturer (SEPPIC Co, France). Water in oil (W/O) emulsion vaccine using Montanide™ ISA 70 VG at a ratio of 30/70 (W/W) aqueous/oil ratio was prepared, taking into consideration that the antigenic content for both antigens not less than 1.4×10^{10} CFU/0.5 ml and not less than 10^8 EID₅₀/0.5 ml for bacterial and viral antigens in the final product.

Quality control of the prepared vaccine

Vaccine's sterility and safety were done according to OIE (2018). A sterility test was done by inoculating one ml of each vaccine on two types of media (Thioglycolate and Bacto-Sabroud Maltose agar) to ensure no growth of bacteria or fungi. Safety test for the inactivated vaccines was performed by inoculating the recommended dose (0.5 ml) intramuscularly of each vaccine into a group of one-day-old ducklings; no adverse effects should be observed during the period of testing.

Experimental design

A total of 148, One-day-old white Pekin ducklings with a history of no vaccination or infection with *R. anatipestifer* or DHV infection were obtained from a private commercial duck farm. The ducklings were provided with recommended feed and management requirements with the maintenance of proper biosecurity. One hundred and thirty-three ducklings were divided randomly into four groups. Group (1), Group (3) and Group (4): has 36 birds per group. Group (2): 25 birds. The remaining 15 ducklings were grouped into three groups (each group includes five ducklings) for the safety test of each prepared vaccine.

Group 1: was vaccinated with *R. anatipestifer* vaccine,

Group 2: was vaccinated with DVH vaccine,

Group 3: was vaccinated with the *R. anatipestifer* and DVH combined vaccine.

Group (4): was left as a negative control.

The vaccinal dose of *R. anatipestifer* vaccines was adjusted to 1.4×10^{10} colony forming unit (CFU)/0.5 ml (Gamal et al., 2021) and the DVH vaccinal dose was 10^8 EID₅₀/0.5ml in all prepared vaccines (Mervat et al., 2005). Half ml of each vaccine was inoculated subcutaneously (S/C) at the dorsal aspect of the duck neck (one day old). Blood samples were collected weekly after vaccination till the eighth week after vaccination). Twenty-two ducks (11 ducklings for RA1 and 11 for RA2) from groups 1, 3 and 4 were challenged 3 weeks post-vaccination against Riemerella.

Serological evaluation of the humoral immune response of ducklings to the prepared vaccines
Indirect Hemagglutination test (IHA)

The IHA test was used to evaluate the humoral immune response against *R. anatipestifer* by glutaraldehyde sheep RBCs (Carter and Cole, 1990).

Serum neutralization test in Vero cell culture

Sera were separated and inactivated in the water bath at 56 C for 20 minutes and tested individually by SNT in VERO cell culture. SNT in VERO cell culture was performed by the microtechnique method as described by Kaleta (1988) in flat bottom tissue culture (TC) microtiter plates. Two-fold dilutions of inactivated ducklings' sera were mixed with equal volumes of virus suspensions containing 100 TCID₅₀ per 0.1 ml. The mixture was incubated at 37° C for 30 minutes. The virus and serum mixture was assayed in VERO cell line culture using 2 wells per dilution. Inoculated cultures and controls were incubated at 37° C for 72 hr. with daily microscopic examination. The endpoint of neutralizing antibody titer was expressed as the reciprocal of the final dilution of serum inhibiting the CPE.

Evaluation of the efficacy of Riemerella vaccines using the Challenge test

Twenty-two ducklings from each vaccinated group with Riemerella (Groups 1 and 3) and 12 ducklings from the control group (Group 4) were challenged with 0.1ml of 10^8 CFU of each *R. anatipestifer* strains RA1 and RA2 intramuscularly (Bebars, 2000). (i.e., For vaccinated groups, eleven ducklings for strain RA1 and eleven for RA2, and control group 6 were for RA1 and 6 for RA2). Ducks after the 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

Data were expressed as mean ± Standard Error (SE). The difference between groups (P Values) was

calculated by one-way ANOVA test using SPSS program version 26 (IBM Corp., 2019).

RESULTS

Quality control testing of the prepared vaccines

- **Sterility test** revealed that all vaccine preparations were found to be free from aerobic and anaerobic bacteria; fungi and mycoplasma.
- **Safety test** confirmed the safety of the three prepared vaccines where all vaccinated ducklings with the double dose did not show any local or systemic post-vaccinal reactions through 15 days of observation.
- **Potency test** was evaluated by applying IHA and SNT on serum samples obtained from vaccinated ducklings in addition to the challenge test against Riemerella.

Indirect Hemagglutination test (IHA) results

As shown in table (1), IHA test was carried out on serum samples obtained from ducklings vaccinated with Riemerella only vaccine (RS), Riemerella and DVH_combined vaccine and control negative group. It was clear that there was no significant difference between both vaccinated groups in all weeks. But there was a significant difference between the vaccinated and control groups. It was observed that in all vaccinated groups, the titers of antibodies increased gradually from the 1st week after vaccination recording their peak by the 6th week ($426.7^a \pm 85.33$ & $341.3^a \pm 85.33$ for the single and combined vaccines, respectively), then began to decline by the 7th week recording their lowest titer for both vaccines ($106.7^a \pm 21.33$).

Table 1: Riemerella IHA antibody titers in vaccinated duckling groups

		Mean RS- IHA titer in duckling groups		
		Group 1	Group 3	Group 4
Weeks after vaccination	1 st	$6.7^a \pm 1.33$	$5.3^a \pm 1.33$	0.0^b
	2 nd	$13.3^a \pm 2.67$	$10.7^a \pm 2.67$	0.0^b
	3 rd	$26.7^a \pm 5.33$	$24^a \pm 8$	0.0^b
	4 th	$53.3^a \pm 10.67$	$48^a \pm 16$	0.0^b
	5 th	$106.7^a \pm 21.33$	$106.7^a \pm 21.33$	0.0^b
	6 th	$426.7^a \pm 85.33$	$341.3^a \pm 85.33$	0.0^b
	7 th	$213.3^a \pm 42.67$	$170.7^a \pm 42.67$	0^b
	8 th	$106.7^a \pm 21.33$	$106.7^a \pm 21.33$	0.0^b

Group 1: vaccinated with the single Riemerella vaccine.

Group-3= vaccinated with the combined vaccine.

Group-4= unvaccinated control group.

*Data are presented as mean ±SE. Means with different superscript small letters indicate significantly different

in the same row between groups at $P < 0.05$ using a one-way ANOVA test.

Serum neutralization test (SNT)

As shown in table (2), SNT test was carried out on serum samples obtained from ducks vaccinated with the single DVH vaccine and those vaccinated with the combined Riemerella and DVH vaccine as well as the negative control group. It was clear that there was no significant difference between the obtained SNT titers for both vaccinated groups in all weeks. But there was a significant difference between the vaccinated and control groups. In all vaccinated groups, detectable DVH antibodies were induced by the 1st week after vaccination till they reached their peak by the 5th week ($128^a \pm 0$) and persisted in 6th week. In the 7th week, DVH combined vaccinated group showed declined antibody titer. In contrast, in the single vaccinated group such titer was stable like the former week and declined by the 8th week for both groups.

Table 2: Mean DVH-SNT antibodies in vaccinated duckling groups

Weeks after vaccination	Mean DH-SNT titer in duckling groups		
	Group 2	Group 3	Group 4
	1 st	18.6±7.06 ^a	24±8.00 ^a
2 nd	37.3±14.11 ^a	26.7±5.33 ^a	0.0 ^b
3 rd	74.7±28.22 ^a	74.7±28.22 ^a	0.0 ^b
4 th	106.7±21.3 ^a	106.7±21.3 ^a	0.0 ^b
5 th	128±0.00 ^a	128±0.00 ^a	0.0 ^b
6 th	128±0 ^a	128±0.00 ^a	0.0 ^b
7 th	128±0.00 ^a	106.7±21.3 ^a	0.0 ^b
8 th	85.3±21.3 ^a	64±0.00 ^a	0.0 ^b

Group-2= vaccinated with the single DVH vaccine.

Group-3= vaccinated with the combined vaccine.

Group-4= unvaccinated control group.

*Data are presented as mean ±SE. Means with different superscript small letters indicate significantly different in the same row between groups at $P < 0.05$ using one-way ANOVA test.

Challenge test

As shown in table (3), the 2 groups vaccinated with Riemerella (Groups 1 and 3) and the negative control group (4) challenged against RA1 and RA2 had

72.7% and 9.1 protection with 10 and 27.3% mortality, respectively.

Table 3: Riemerella challenge test

Parameter	Duckling groups					
	Group-1		Group-3		Group-4	
	RA1	RA2	RA1	RA2	RA1	RA2
Mortality No.	3	3	3	3	10	10
Mortality %	27.3	27.3	27.3	27.3	90.9	90.9
Protection %	72.7	72.7	72.7	72.7	9.1	9.1

Group-1= vaccinated with the single RS vaccine.

Group-3= vaccinated with the combined vaccine.

Group-4= unvaccinated control group.

DISCUSSION

Riemerella vaccine, DVH vaccine and combined Riemerella - DVH vaccine were prepared adjuvanted with Montanide™ ISA 70 VG. Ducks were grouped and vaccinated with a single dose of each type of vaccine according to their group. The successfully combined vaccines decrease the stress on birds and laborers and save time and cost.

The usage of IHA for evaluating the antibody titers against Riemerella in this study, as shown in table (1), revealed that the antibody titers increased gradually from the 1st week after vaccination in both vaccinated groups by Riemerella single and combined vaccine as (6.7 and 5.3 respectively) till the 6th week reaching their highest value to be 426.7 and 341.3 respectively. By the 7th week, these titers began to decline (213.3 for RS group and 170.7 for the combined one). In the 8th week, the antibody titers reached their lowest values (106.7 for both RS group and the combined one). Statistically, there was no significant difference between both vaccinated groups in the same week for all weeks. Still, there was a significant difference between the vaccinated and control groups. **Gamal et al., (2021)** agreed with the current study in the indications of IHA results, as their results for Riemerella single vaccine group and the Riemerella and avian influenza combined vaccine increased till the 6th week and then decreased from the 7th week. The obtained results in table (1) are in agreement with **Zhang et al., (2014)**, who reported the highest antibody titers against *R.*

anatipestifer were at 37 and 44 days (around 6 weeks) post-immunization.

The usage of SNT for evaluating the antibody titers against DVH in this study revealed as shown in table (2), that the antibody titers increased gradually from 1st week after vaccination in both vaccinated groups (DS and Combined; 18.6, 24 respectively) till the 5th week. The titers persisted at 128 at weeks 5th and 6th for both vaccinated groups and week 7 for DS group. On the other hand, the combined vaccine group started to decrease from 7th week (106.7) to (64) in 8th week. But the start of decreasing for DS vaccine group was at 8th week (85.3).

These results agreed with those obtained by **Mervat et al., (2000)** who concluded that the DVH vaccine gives a higher level (128) of immunity in the vaccinated group with DVH, from the 5th week after the vaccination till the 7th week. Also, **Mervat et al., (2005)** reported that the 7th week was the highest antibody titers against DVH in the monovalent DVH vaccine group (173.6) and the combined DVH and duck plague vaccine (149.3). In the 8th week, the titers declined for the monovalent one (64), but stabled for the combined one (149.3). According to Mervat (1997), the protective SNT titer against DVH is 32, so the prepared vaccines are protective against DVH till the 8th week.

For the two Riemerella-prepared vaccines, when evaluated by the challenge test against Riemerella as shown in table (3), the vaccines outperformed the infection by giving 72.7% protection with 27.3% mortality. On the contrary, the control group had 9.1% natural protection with 90.9% mortality. **Gamal et al., (2021)** when preparing the Riemerella vaccine single and combined adjuvanted with Montanide™ ISA 71 VG for both vaccines and challenged the vaccinated groups 3 weeks post-vaccination with Riemerella, the protection against Riemerella single vaccine was 70% and for the combined vaccine was 75% but for the control was 20%. **Zhang et al., (2014)** cited that the protection level for ducklings immunized with Riemerella oil emulsion vaccine was only 69.2%.

But all of the ducks died after the challenge in the control group. **Stoute et al., (2016)** reported that a prepared *R. anatipestifer-E. coli* O78 bacterin induced 70 to 100% protection in challenged experimental groups and the mortality rates for control groups were 50 to 100% according to the challenge time. **EL-Rawy et al., (2020)** also reported that the protection rate for Riemerella oil adjuvanted vaccines either monovalent or combined with duck Pasteurellosis 3 weeks post booster dose ranged from 80 to 100% protection. According to Aboul Saoud (2010), the protective IHA

titer against Riemerella is 128, so the prepared vaccines are protective against Riemerella till the 7th week.

CONCLUSION

R. anatipestifer and duck viral hepatitis are the most economic diseases of ducks. The RS and DH combined vaccine is effective and easier to manipulation than every single one. Also, the current study proved that the combination in this vaccine had no adverse effect on the antibody titers compared to the titers obtained with the separate single vaccines. A single dose of that prepared vaccine is enough to cover the susceptible age of both diseases in young ducklings, especially in broiler duck flocks.

Conflict of interest

The authors declare that there is no conflict of interest for this publication.

Author's contribution

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

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