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Preparation and evaluation of combined oil adjuvant vaccine against duck pasteurellosis and *Riemerella anatipestifer* infection in ducks

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Abstract
Single and inactivated combined vaccines were prepared against duck Pasteurellosis and *Riemerella anatipestifer* infection in ducks. All the prepared vaccine were checked for sterility and found to be free from foreign contaminants. Also, all were safe for vaccinated birds (showing no post vaccination sickness) and were immunogenic inducing high levels of specific antibodies against both of *P. multocida* (A5 and D2) and *R. anatipestifer* strains (RA1 and RA2). Indirect hemagglutination (IHA and ELISA techniques) revealed that such vaccines induced a satisfactory antibody response when used as primary vaccination ducks. Improved antibody response was recorded till 6 month when the vaccines were given in two doses at three weeks intervals. There is no antagonizing effect between the used antigens in the combined vaccine on the duck's immune responses. The protective potential efficacy of the prepared vaccines was also measured by determining the rate of protection of the birds of each vaccinated group by challenge exposure at 3 weeks post booster vaccination.

Keywords: duck pasteurellosis, *Riemerella anatipestifer* infection

Introduction
Duck cholera and *Riemerella anatipestifer* infection (RA) causes major economic losses in the duck, through high mortality rates, poor feed conversion, increased condemnations, and high treatment costs (Kardos *et al.,* 2007; Sun *et al.,* 2012) [12, 19].
Infections with *Pasteurella multocida* in duck are extremely common across the world. This organism is very common in Asia and the Middle East countries. Signs in acute outbreaks can be just sudden death in large number of birds; however, in chronic infections, signs of depression, conjunctivitis and dyspnea can occur.
Riemerella anatipestifer causes disease in ducks throughout the world. Formerly known as Pasteurella anatipestifer, this organism 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 saculitis with a casious deposit can be found (Sandhu, 1986; Higgins *et al.,* 2000) [17, 11]
Various antibiotics are currently used to prevent and control the infection in ducks, but they accelerate the emergence of drug-resistant strains (Chen *et al.,* 2010; Chen *et al.,* 2012) [5, 6]. The resistance of *Pasteurella multocida* and *R. anatipestifer* too many antibiotics has increased greatly, and antibiotic residues have been detected in duck-related products (Sun *et al.,* 2012) [19]. 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) [16].
The development of a combined inactivated vaccine 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.

So this study was aimed to develop a combined vaccine against Duck Pasteurellosis and *Riemerella anatipestifer* infection and to evaluate both the protection of vaccinated ducks from challenge and their immune responses after vaccination. *Pasteurella multocida* serotypes A and D and *Riemerella anatipestifer* strains serotypes RA1 and RA2 are responsible for most of the major outbreaks in Egypt were selected for use in developing the vaccine.

Materials and Methods

Bacterial strains

a) ***Pasteurella multocida* serotypes:** *Pasteurella multocida* serotype A5 and D2 were supplied by Aerobic Bacterial Vaccine Department, VSVRI, Abbassia, and Cairo.

b) ***Riemerella anatipestifer* isolates:** All *Riemerella anatipestifer* isolates RA1 and RA2 were previously isolated from naturally occurring outbreaks on commercial Pekin duck farms in Egypt and identified morphologically, biochemically and molecularly characterization in aerobic bacteria.

Vaccine preparation

It was prepared according to (Pathanasophon *et al.,* 1996) [15]

Strains were cultured separately in Tryptic Soya Broth (TSB) at 37 ºC for 24 hours with shaking. The count of bacterial CFU for each strain was adjusted to1.4x 10^{10} CFU/ml for *R. anatipestifer*, and 3.25x10^{10} CFU for *P. multocida* serotypes. The bacteria were then inactivated with 0.5% formaldehyde at 37 ºC for 24 hours. The combined vaccine was made by blending 1 volume each of inactivated strains and 7 volume of Montanide ISA 70 VG adjuvant according to the manufacturer's protocol.

Quality control of the prepared vaccine

The prepared vaccines were tested for sterility and safety following the standard international protocols as described (Code of federal regulation, 2017 and OIE 2019).

Experimental design

One-day-old white Pekin duckling with history of no vaccination or infection with fowl cholera or *Riemerella anatipestifer* infection were obtained from a duck flock breed at a private commercial duck farm. For this experiment, the birds were reared in special isolators and were provided with recommended feed and other management requirements with maintenance of proper biosecurity. The ducks used for the experiments were grouped as shown in Table 1.

Table 1: Experimental design

Group	1	2	3	4
Type of vaccine	***R. anatipestifer* bivalent vaccine**	***P. multocida* bivalent Vaccine**	**Combined vaccine of *R. anatipestifer* and P. multocida**	**Control non-vaccinated**
Dose/route of vaccination	0.5 ml S/C at upper dorsal part of the neck			
1st dose	At 2-3 weeks of age			
2nd dose	At 4-6 weeks of age			
Blood s sampling	Blood sample were collected 2 weeks intervals till 6 months for evaluation of humoral immunity by IHA and ELISA			
Challenge test	All groups of ducks received 0.1 ml of the virulent strains of RA (RA1,RA2) and *P. multocida* (5A, D2)			

Serological tests for evaluation of humoral immune response

Blood samples were collected aseptically after 2 weeks of first vaccination and each 2 weeks till 6 month of age for evaluation of humeral immunity by IHA and ELISA techniques.

1. **Indirect hemagglutination test (IHA):** Glutaraldehyde RBCs and capsular antigen of *R. anatipestifer* and *P. multocida* were prepared according to (Carter and Cole, 1990) [4]. A vaccine is considered efficient as induce sera conversion in the sera of vaccinated ducks.

2. **Enzyme linked immunosorbent assay (ELISA):** It was done according to (Burgess, 1988) [3]. The antibodies titers against *R. anatipestifer* and *P. multocida* in the sera of duck were obtained by the cut off absorbance value were 0.55. The above reading of the cut off value of serum sample was regarded as positive.

Challenge test

All the vaccinated and control group of ducks were subjected to challenge subcutaneously with 0.2 ml of virulent isolates of *Pasteurella multocida* serotypes A and D and *Riemerella anatipestifer* isolates serotypes RA1 and RA2, three weeks of booster with 100 LD50 (Babars, 2000) [2].

Post-challenge observation of birds: Birds after challenge were observed daily for a week for any mortality and or clinical signs and symptom. The clinical findings of both the vaccinated and unvaccinated birds were observed and recorded.

Results and Discussion

Ducks, just like any other poultry species, are also prone to diseases and infections. When rearing ducks, precautionary measures should be taken to prevent them from contracting diseases, and if they are already infected, prevent them from spreading further infection. Since ducks live in close proximity with each other, the prime important measure is that ducks should be vaccinated to prevent these infections (Christensen *et al.,* 2008) [8].

Table (2) showed overall mean of antibodies titer against *R. anatipestifer* was 112 and 48 in the group of ducks vaccinated with 2 isolates of *R. anatipestifer* but were 320 and 122 in the group of ducks vaccinated with combined inactivated oil vaccine against *R. anatipestifer* and *P. multocida* as well as increases the titer of the overall mean of antibodies against *P. multocida* in the combined vaccinated duck with *R. anatipestifer* and *P. multocida* were 213 and 170 compared to117 and 72 in the vaccinated group of ducks with *P. multocida* only. The peak antibodies titers were in the combined vaccinated duck group was at the intervals of 6 and 8 weeks post the 1st vaccination. These data were agree with (Pathanasophon *et al.,* 1996) [15], who regarded that the antibody titer were higher at 3,4 weeks post 1st vaccination

the peak of antibody titer was at 6-8 weeks post booster vaccination.

Table (3) revealed the overall mean antibodies titer against *R. anatipestifer* and *P. multocida* estimated by ELISA test, in the group of ducks vaccinated with combined vaccine were 4292, 4607and 3195, 4149 compared with 3407, 3392 and 2696, 3480 in the 2 group of ducks vaccinated with *R. anatipestifer* RA1 and RA2 and *P. multocida* type D2 and 5A. The peak antibodies titer against *R. anatipestifer* and *P. multocida* continue from 6 weeks till 12 weeks post the 1st vaccination these data where in the same manner of (Hatfield *et al*., 1987) [10] who described that ELISA was more sensitive than agglutination test to detect serum antibodies in ducks. Inactivated bacterins have been reported to prevent or reduce mortality due to *R. anatipestifer* (Layton and Sandhu, 1984) [13] because immunity induced by bacterins is serotype specific ideal bacterins should contain cells of the predominant serotypes to provide an effective protection (Timms and Marshall, 1989) [20] were using ELISA and found low antibody response in some birds following vaccination at 7 days of age suggested that immunity of immune system interfere with maternal immunity. (Sandhu and Leister, 1991) [18] Obtained better immune response post inoculated duckling at age 2-3 weeks with inactivated trivalent *R. anatipestifer* vaccine into inoculation.

The data in Table (4), illustrated that the protection percentage against the challenge with virulent strain of *R. anatipestifer* type 1 (RA1) was 80% for *R. anatipestifer* vaccine and 100% for combined vaccine in compared with 0% for control group. While type (RA2) was 90% for *R. anatipestifer* vaccine and 100% for combined vaccine in compared with control group. The data in table (5), illustrated that the protection percentage against the challenge with virulent strain of *P. multocida* type (A) was 90% for *P. multocida* vaccine and 100%for combined vaccine in compared with 0% for control group. While type the protection percentage against challenge with virulent strains of *P. multocida* (D) was 80% for *P. multocida* vaccine and 100% for combined vaccine in compared with control group. These results were in the same manner with that of (Pathanasophon *et al.*, 1996) [15].

From the above mentioned results, it can deduced that combination of *R. anatipestifer* and *P. multocida* antigens has no adverse effect on the humoral immune response of vaccinated ducks as detected by IHA and ELISA tests to either of them separately. No mutual interference among the four antigens could be observed by detecting the antibody titers to both of them. The obtained results agree with those reported by (Derieux and Dick 1980; Nawath and Ayolla 1981) [9, 14] who mentioned that viral vaccines of poultry did not interfere with the immune response of birds to bacterial vaccines if both are given in a combined form.

In conclusion, a safe combined duck Pasteurellosis and *Riemerella anatipestifer* inactivated vaccine was developed successfully. The produced vaccine is safe, sterile and provided an effective protection of duck against challenges with virulent *Pasteurella multocida* serotype A and D and *Riemerella anatipestifer* isolates RA1 and RA2. This study recommend the production and commercial use of such vaccine for the effective control of duck Pasteurellosis and *Riemerella anatipestifer* infection in ducks in Egypt.

Table 2: Level of IHA antibody titers in sera of duckling following vaccination with *R. anatipestifer* and *P. multocida*

Interval of serum collection	**Type of vaccinated groups**								
Pre-vaccination	**Monovalent *R. anatipestifer***		**Monovalent P. multocida**		**Combined vaccination with R. antipestifer and P. multocida**				**Control**
	RA1	RA2	5A	D2	RA1	RA2	5A	D2	
	2	2	2	4	2	4	2	2	2
Primary vaccination									
2nd weeks	32	32	64	16	64	256	64	64	2
4th weeks	64	32	64	32	256	128	64	256	2
Booster vaccination									
6th weeks	256	64	128	64	256	128	128	256	4
8th weeks	128	64	64	128	1024	128	256	256	2
10th weeks	128	64	256	128	256	64	256	128	4
12th weeks	64	32	128	64	64	32	512	64	2
Overall mean	112	48	117	72	320	122	213	170	3

Table 3: Level of ELISA antibody titers in sera of duckling following vaccination with *R. anatipestifer* and *P. multocida* vaccines

Interval of serum collection	**Type of vaccinated groups**								
Pre-vaccination	**Monovalent R. anatipestifer**		**Monovalent P. multocida**		**Combined vaccination with R. antipestifer and P. multocida**				**Control**
	RA1	RA2	5A	D2	RA1	RA2	5A	D2	
	140	140	120	130	120	140	130	120	120
Primary vaccination									
2nd weeks	1300	1400	1211	1211	2100	2710	1300	2040	176
4th weeks	1688	1715	2042	1688	3100	3490	1100	3546	234
Booster vaccination									
6th weeks	3236	3680	2602	3100	4002	4116	3010	3998	180
8th weeks	4599	4560	3236	4010	5871	5346	4562	5010	310
10th weeks	6077	4998	4998	5871	6120	6110	4303	5300	234
12th weeks	3546	4003	2090	5001	4560	5871	4900	4998	175
Overall mean	3407	3392	2696	3480	4292	4607	3195	4149	218

Table 4: Results of challenge test against *R. anatipestifer* type (RA1 and RA2) in ducks vaccinated with combined vaccine of *R. anatipestifer* and *P. multocida* vaccine and monovalent *R. anatipestifer* vaccine

Type of vaccine	Monovalent *R. anatipestifer*		Combined vaccine of R. anatipestifer and P. multocida		Control group (non- vaccinated)	
	(RA1)	(RA2)	RA1	RA2	RA1	RA2
Total no. of ducks	10	10	10	10	10	10
Dead	2	1	0	0	10	10
Survived	8	9	10	10	0	0
Protection %	80%	90%	100%	100%	0%	0%

Table 5: Challenge test against *P. multocida* in ducks vaccinated with combined vaccine of *R. anatipestifer* and *P. multocida* and monovalent *P. multocida* vaccine

Type of vaccine	Monovalent *P. multocida* vaccine		Combined vaccine of R. anatipestifer and P. multocida		Control group (non- vaccinated)	
	5A	D2	5A	D2	5A	D2
Total no. of ducks	10	10	10	10	10	10
Dead	1	2	0	1	10	10
Survived	9	8	10	9	0	0
Protection %	90%	80%	100%	100%	0%	0%

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