**Original Paper**

Enhancing immunogenicity and protection against *Riemerella anatipestifer* in ducks: Comparative evaluation of adjuvanted inactivated vaccines

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Riemerella anatipestifer infections impose significant economic burdens on duck production globally. Addressing the challenges posed by diverse serotypes, limited cross-protection, and varying field outbreaks, this study aimed to evaluate the safety, efficacy, and immunogenicity of three inactivated *R. anatipestifer* (R.A.) vaccines adjuvanted with montanide ISA70 oil, paraffin oil, and Salmonella Typhimurium lipopolysaccharide (S. Typhimurium LPS). The vaccines were administered to ducks, and their immune responses were assessed. The cellular immune response was measured through nitric oxide levels, while the humoral immune response was evaluated using the indirect hemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA). Challenge tests were conducted against virulent R.A. strains to determine protection percentages. The results indicated that the S. Typhimurium LPS-adjuvanted vaccine elicited the highest nitric oxide levels (150.23 $\mu\text{mol/mL}$), IHA titers (550), and ELISA levels (2.9g/dL). This group demonstrated 100% protection postchallenge, while the montanide ISA70 oil and paraffin oil-adjuvanted vaccines showed 95% and 85% protection, respectively. Clinical signs and postmortem lesions were markedly reduced in vaccinated groups compared to the control positive group. The study underscores the potential of the S. Typhimurium LPS-adjuvanted R.A. vaccine in inducing robust immune responses and conferring significant protection against prevalent serotypes 1 and 2. This research offers insights into improving duck septicemia control strategies and emphasizes the importance of tailored vaccine formulations for enhanced protection against *R. anatipestifer* infections in duck populations.

1. INTRODUCTION

Riemerella anatipestifer, classified as a gram-negative, rod-shaped, nonmotile, and non-spore-forming bacterium (Sandhu, 2008), inflicts substantial economic losses in the global and Egyptian duck production sectors (El-Hamid et al., 2019). This infectious scourge induces devastating consequences, characterized by heightened mortality rates, condemnations, compromised feed conversion rates, and exorbitant treatment expenses (Kardos et al., 2007). Notably, among ducks younger than seven weeks old on affected farms, *R. anatipestifer* infections drive mortality rates to a concerning range of 10% to 75% (Subramaniam et al., 2000). The pathogenicity of *R. anatipestifer* is compounded by the presence of approximately 21 identified serotypes globally, exhibiting poor cross-protection among these variants (Kardos et al., 2007; Sandhu, 2008). The prevalence of major outbreaks worldwide and in Egypt is primarily attributed to serotypes 1, 2, and 10 (Hu et al., 2001), further complicating disease control and management strategies. The escalating emergence of drug-resistant *R. anatipestifer* strains is a pressing concern, propelled by the indiscriminate use of a wide array of antibiotics (Chen et al., 2010, 2012). This resistance surge poses a critical challenge, accentuated by the detection of antibiotic residues in duck-derived

products (Sun et al., 2012). Consequently, vaccination emerges as the most promising strategy for disease control, particularly owing to the limitations of vaccines founded on a single serotype of killed bacteria. These vaccines have shown a dearth of significant cross-protection capabilities (Layton & Sandhu, 1984; Sandhu, 1979). Immunogenic studies focusing on the outer membrane protein A (OmpA) of *R. anatipestifer* have highlighted its potential, albeit subunit vaccines derived from it demonstrating inefficacy against infections by heterologous-serotype strains (Hu et al., 2001; Huang et al., 2002; Shunzhou et al., 2002). Similarly, the use of recombinant *R. anatipestifer* GroEL has exhibited partial cross-protection between serotypes 1 and 2, falling short of providing complete protection (Han et al., 2012). Thus, the urgent need to develop an effective *R. anatipestifer* vaccine encompassing the most prevalent serotypes within the geographical area is paramount for robust disease prevention. Studies showcasing the effectiveness of lipopolysaccharide (LPS) in augmenting antibody activity in the serum of immunized birds and elevating nitric oxide levels underscore its potential in vaccine development (De Boever et al., 2008; Khatsenko et al., 1993; Son & Kim, 1995; Sunwoo et al., 1996). This experiment aims to produce a bivalent *R. anatipestifer*-killed

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vaccine integrated with various adjuvants. Furthermore, it seeks to evaluate the efficacy of the vaccine in protecting ducks against serotypes 1 and 2—recognized as major outbreak-inducing serotypes. Additionally, the study endeavors to assess both the cellular and humoral immune responses postvaccination and determine the vaccine's efficacy in preventing mortality.

2. MATERIAL AND METHODS

2.1. *Riemerella anatipestifer* Isolates: Serotypes 1 and 2

Drawing from our prior investigations, a comprehensive study regarding *R. anatipestifer* prevalence across five governorates in Egypt (Menofya, Qalubia, Gharbia, Sharkia, and Behara) was conducted and indicated the predominance of serotype 1 and serotypes 2 strains. Consequently, these strains were employed in the production of a bivalent vaccine utilizing various adjuvants. Furthermore, these strains were employed as the challenge strains throughout the study

2.2. Lipopolysaccharide (LPS) Extraction and Purification from *S. Typhimurium*

Lipopolysaccharide extraction and purification from *S. Typhimurium* were performed in accordance with the method described by Hassan et al., (2020). Initially, *S. Typhimurium* colonies were suspended in peptone water and subjected to ultracentrifugation at 10,000× g for 5 min. Subsequently, the resulting pellets were washed twice with pH 7.2 phosphate-buffered saline (PBS), while the supernatant was discarded. The weight of the obtained *S. Typhimurium* pellets from the three extraction methods remained consistent at 10 mg each. The pellets (10 mg) were then resuspended in a 0.5 mL mixture comprising 1 mol/mL propanol and sodium hydroxide (NaOH) (in a ratio of 5:3, v:v) with an alkaline pH of 11.4. This suspension was maintained in a firmly closed Eppendorf tube within a 20°C water bath for 2h while being mildly stirred using a magnetic stirrer. The resultant mixture was subsequently cooled at -20°C and subjected to ultracentrifugation at 10,000× g for 15 min. The supernatant was carefully collected, and the gel-like sedimented layer was re-extracted using 312.5 µg of isopropanol before undergoing another round of ultracentrifugation at 10,000× g for 5 min. The discarded precipitate was eliminated, and the supernatant was combined with the previously collected supernatant, diluted using an equal volume of distilled water, and centrifuged at 2000 rpm for 5 min. The resulting supernatant was collected for further analysis.

2.3. Preparation of Inactivated Bivalent *Riemerella anatipestifer* Vaccines

The preparation of inactivated bivalent *Riemerella anatipestifer* vaccines was conducted according to the method outlined by Wang et al., (2012). Initially, suspensions containing two serotypes of *Riemerella anatipestifer* (serotypes 1 and 2) were prepared, each containing 1.5×10^{10} CFU. The bacterial colony forming unit (CFU) count for each strain was determined by measuring the optical density at 600 nm (OD₆₀₀), where an OD₆₀₀ of 1 equates to 2.5×10^9 CFU of bacteria. The suspensions of the two serotypes were inactivated using 0.5% formalin at 37°C for 24 hours. Following complete inactivation, equal volumes of the inactivated suspensions were thoroughly mixed together. The resultant mixture was preserved with 0.01% thiomersal then mixed thoroughly with mineral oil either paraffin oil or montanide ISA70 and *Salmonella Typhimurium* lipopolysaccharide in a

percentage of (1:1) oil: culture. Each duck was subcutaneously inoculated in the neck region with 0.5 ml of the vaccine preparation

2.3.1. Sterility Test

The prepared vaccines underwent rigorous sterility testing to ascertain their freedom from any contamination. The testing procedure involved the inoculation of thioglycollate broth (Oxoid), nutrient agar (Oxoid), and MacConkey agar media (Oxoid) with the vaccine, followed by incubation at 37°C for 72 hours. Furthermore, inoculation was performed on Sabouraud dextrose agar (Oxoid) plates and incubated at 25°C for 14 days. Additionally, mycoplasma broth (Oxoid) and agar (Oxoid) plates were inoculated and incubated at 37°C for 72 hours and 14 days in a 5% CO₂ environment. No observable growth was evident on any of the inoculated media, indicating the successful sterile vaccine of *Riemerella anatipestifer*.

2.3.2. Safety Test

To evaluate safety, twenty mice were subcutaneously injected with 0.2 ml of the prepared vaccines. The mice were continuously observed for duration of 7 days for any signs of local reactions, clinical symptoms, or mortality.

2.3.3. Potency Test

Potency assessment involved vaccination followed by challenge experiments.

2.4. Experimental Design and Challenge Test

2.4.1. Experimental Design

The experiment aimed to assess the immune response of different adjuvants in bivalent inactivated vaccines and determine their protective efficacy against *Riemerella anatipestifer* (R.A.) challenge.

2.4.1.1. Experimental Animals: Ducklings

A total of 160 one-day-old Pekin ducklings were procured from a commercial supplier and maintained within an optimal temperature range of 28 to 30°C throughout the study. The ducklings were accommodated in cages with continuous access to ample food and water during the experimental period

2.4.1.2. Duration and Subjects

The experiment spanned 12 weeks. The ducklings were divided into five groups as follows: G1: Control negative, G 2: Control positive, G 3: Ducks vaccinated with killed R.A. Vaccine using mentioned oil (first dose at 2 weeks old, booster at 6 weeks old, administered via 0.5 cm subcutaneous injection), G 4: Ducks vaccinated with killed R.A. Vaccine using paraffin oil (same dosing regimen as G 3), and G 5: Ducks vaccinated with killed R.A. Vaccine using *S. Typhimurium* LPS (same dosing regimen as G 3)

2.4.2. Challenge Test

2.4.2.1. Challenge Strains

Riemerella anatipestifer strains serotype 1 and serotype 2 were employed. The median lethal dose (LD₅₀) for each strain was previously determined as 4.74×10^6 CFU/mL and 1.07×10^5 CFU/mL, respectively, as per (Wang et al., 2012)

2.4.2.2. Challenge Procedure

At 12 weeks old, all duck groups except for G 1 were subjected to subcutaneous injection of serotype 1 and serotype 2 strains at a dose of 2 LD₅₀ in 0.5 ml saline. Daily

monitoring was conducted for 7 days post-challenge to record the clinical signs and mortality rates.

2.4.2.3. Sample Collection

Blood samples were gathered to assess cellular immunity via the macrophage activity test (nitric oxide conc.). Serum samples were collected to evaluate humoral immunity using ELISA and IHA techniques. Further blood and serum samples were obtained after the challenge to assess cell-mediated and humoral immunity

2.5. Determination of the immune response to the prepared experimental vaccines

2.5.1. Evaluation of Cellular Immune Response

Macrophage Activity Test (Nitric Oxide Conc.): Cellular immunity in vaccinated and unvaccinated ducklings was monitored at regular intervals post-vaccination and challenge. The nitric oxide concentration in the macrophage supernatant was assessed as an indicator of the cellular immune response. The method was based on Rajaraman et al., (1998)

2.5.2. Evaluation of Humoral Immune Response

Indirect Hemagglutination Test (IHA): The effect of the bivalent inactivated vaccine with different adjuvants on humoral antibody titers was assessed using IHA. Evaluation of humoral immune response based on Carter and Rappy (1962) Carter and Cole (1990) methods. Enzyme-Linked Immunosorbent Assay (ELISA): Similar evaluation of humoral antibody titers as affected by the bivalent inactivated vaccine with different adjuvants was performed using ELISA. Evaluation of humoral immune response was based on the method developed by Briggs and Skeeles (1984).

2.6. Statistical analysis

Data and results were collected and computed using Microsoft Excel 2016. To analyze the data, we utilized the Statistical Package for Social Sciences software, specifically version 25.0 (SPSS Inc., Chicago, IL). We computed descriptive statistics, including means and standard errors, for each group and parameter. To compare the parameters

among the various groups, we employed a one-way analysis of variance (ANOVA). Post-hoc tests, such as Tukey's or Dunn's test, were performed for pairwise comparisons if significant differences were detected (Campbell, 2021)

3. RESULTS

3.1. Safety and Sterility of Prepared Vaccines

No observable growth was evident on any of inoculated media, indicating the successful sterile vaccine of *Riemerella anatipestifer*

3.2. Cellular Immune Response (Nitric Oxide Levels) - Macrophage Activity Test (Table 2)

Nitric oxide levels were assessed in ducks vaccinated with different adjuvanted R.A. vaccines. The vaccine adjuvanted with *S. Typhimurium* LPS exhibited the highest nitric oxide levels (150.23 $\mu\text{mol/ml}$), followed by montanide ISA70 oil (110.20 $\mu\text{mol/ml}$) and paraffin adjuvanted vaccine (70.41 $\mu\text{mol/ml}$).

3.3. Humoral Immune Response Evaluation by IHA and ELISA (Tables 3 and 4)

Antibody titers were determined using the Indirect Hemagglutination Test (IHA) and Enzyme-Linked Immunosorbent Assay (ELISA). The *S. Typhimurium* LPS adjuvanted vaccine displayed the highest antibody titers (IHA: 550; ELISA: 2.9g/dL), followed by montanide ISA70 oil (IHA: 512; ELISA: 2.4g/dl) and paraffin adjuvanted vaccine (IHA: 256; ELISA: 1.7g/dl)

3.4. Challenge Test Results against Virulent R.A. Strains

The protection percentage after challenge with serotypes 1 and 2 was as follows: Group 5 (vaccinated with R.A. vaccine adjuvanted with *S. Typhimurium* LPS) had 100% protection. Group 3 (vaccinated with R.A. vaccine adjuvanted with montanide oil) exhibited 95% protection. Group 4 (vaccinated with R.A. vaccine adjuvanted with paraffin oil) showed 85% protection. The control positive group (Group 2) displayed severe clinical signs of duck septicemia and pronounced postmortem lesions compared to the vaccinated groups

3.5. Clinical Signs and Postmortem Lesions

Severe clinical signs and postmortem lesions (Fig. 1) were observed in the control positive group, including respiratory and nervous manifestations, while vaccinated groups showed minimal clinical symptoms and lesions

Table (1): Evaluation of Cellular Immunity by Detection of Nitric Oxide Concentration in Macrophage Supernatant (Nitric Oxide Levels): (Mean \pm SEM) and (N=5)

Intervals times of blood collection	Control		Groups of ducks vaccinated with different adjuvanted RA vaccine		
	G 1	G 2	G 3	G 4	G 5
Before vaccination	7.12 \pm 0.43 ^a	7.17 \pm 0.43 ^a	7.56 \pm 0.67 ^a	7.94 \pm 0.33 ^a	7.33 \pm 0.22 ^a
1 st vaccination at 2 weeks old					
2ws post 1st vaccination	7.12 \pm 0.43 ^a	7.12 \pm 0.43 ^a	21.59 \pm 0.79 ^b	20.50 \pm 0.62 ^b	23.53 \pm 0.98 ^c
4ws post 1st vaccination	7.12 \pm 0.43 ^a	7.12 \pm 0.43 ^a	50.16 \pm 0.1.02 ^d	25.77 \pm 0.61 ^c	53.57 \pm 0.51 ^e
Booster vaccination at 6 weeks old					
7ws post 1st vaccination	7.12 \pm 0.43 ^a	7.12 \pm 0.43 ^a	57.57 \pm 0.65 ^d	35.33 \pm 0.67 ^c	60.20 \pm 3.19 ^f
10ws post 1st vaccination	7.12 \pm 0.43 ^a	7.12 \pm 0.43 ^a	110.20 \pm 1.30 ^d	70.41 \pm 0.69 ^c	150.23 \pm 0.12 ^e
Challenge at 12 weeks old					
3 days post challenge	7.12 \pm 0.43 ^a	90.20 \pm 1.30 ^d	100 \pm 2.50 ^d	40.20 \pm 1.30 ^d	105.20 \pm 1.30 ^d

G 1: Negative control, G 2: Positive control, G 3: Ducks vaccinated with killed *R.A* vaccine with mentioned oil, G 4: Duck vaccinated with killed *R.A* Vaccine with paraffin oil, G 5: Duck vaccinated with killed *R.A* vaccine with *S. Typhimurium* LPS. ws: weeks. Means with different superscript letters in the same row differ significantly at $P < 0.05$

Table (2): Overall Mean Antibody Titers in Duck Groups Vaccinated with Various Adjuvanted R.A. Inactivated Vaccines Using IHA Technique (Mean ± SEM) and (N=5)

Intervals times of serum collection	Control		Groups of ducks vaccinated with different adjuvanted R.A vaccine		
	G1	G 2	G 3	G 4	G 5
Pre vaccination	2	3	2	2	2
1 st vaccination at 2 weeks old					
2ws post 1st vaccination	2	3	64	32	128
4ws post 1st vaccination	2	2	128	64	256
Booster vaccination at 6 weeks old					
7ws post 1st vaccination	2	2	256	128	512
10ws post 1st vaccination	2	2	512	256	550
Challenge at 12 weeks old					
3days post challenge	2	200	450	240	490
Overall mean	2	35	235	120	323

G 1: Negative control, G 2: Positive control, G 3: Ducks vaccinated with killed R. A vaccine with mentioned oil, G 4: Duck vaccinated with killed R. A Vaccine with paraffin oil, G 5: Duck vaccinated with killed R. A vaccine with S. Typhimurium LPS.ws: weeks. Means with different superscript letters in the same row differ significantly at P< 0.05

Table (3): Overall Mean Antibody Titers in Duck Groups Vaccinated with Different Adjuvanted R.A. Killed Vaccine Using ELISA Technique (Mean ± SEM) and (N=5)

Intervals times of serum collection	Control		Groups of ducks vaccinated with different adjuvanted R.A vaccine		
	G1	G 2	G 3	G 4	G 5
Pre vaccination	0.4	0.4	0.4	0.5	0.4
1 st vaccination at 2 weeks old					
2ws post 1st vaccination	0.4	0.4	1	0.7	1
4ws post 1st vaccination	0.4	0.4	1.5	1.2	1.8
Booster vaccination at 6 weeks old					
7ws post 1st vaccination	0.4	0.4	2.2	1.5	2.7
10ws post 1st vaccination	0.4	0.4	2.4	1.7	2.9
Challenge at 12 weeks old					
3days post challenge	0.4	1.5	2	1.4	2.4
Overall mean	0.4	0.6	1.6	1.2	1.9

G 1: Negative control, G 2: Positive control, G 3: Ducks vaccinated with killed R. A vaccine with mentioned oil, G 4: Duck vaccinated with killed R. A Vaccine with paraffin oil, G 5: Duck vaccinated with killed R. A vaccine with S. Typhimurium LPS.ws: weeks. Means with different superscript letters in the same row differ significantly at P< 0.05

Table (4): Challenge Test Results of Vaccinated Duck Groups with Different Adjuvants against Virulent Strains of RA1 and RA2 (Mean ± SEM) and (N=5)

Types of R.A virulent serotype	Challenge dose	Control	Groups of vaccinated ducks with different adjuvanted RA vaccines				
			G1	G2	G3	G4	G5
R.A serotype 1	No. of ducks	10	10	20	20	20	
	Survive	10	2	19	17	20	
	Dead	0	8	1	3	0	
	Protectio n %	100	20	95	85	100	
		%	%	%	%	%	
R.A serotype 2	No. of ducks	10	10	20	20	20	
	Survive	10	1	19	18	20	
	Dead	0	9	1	2	0	
	Protectio n %	100	10	95	90	100	
		%	%	%	%	%	

G 1: Negative control, G 2: Positive control, G 3: Ducks vaccinated with killed R. A vaccine with mentioned oil, G 4: Duck vaccinated with killed R. A Vaccine with paraffin oil, G 5: Duck vaccinated with killed R. A vaccine with S. Typhimurium LPS.ws: weeks. Means with different superscript letters in the same row differ significantly at P< 0.05

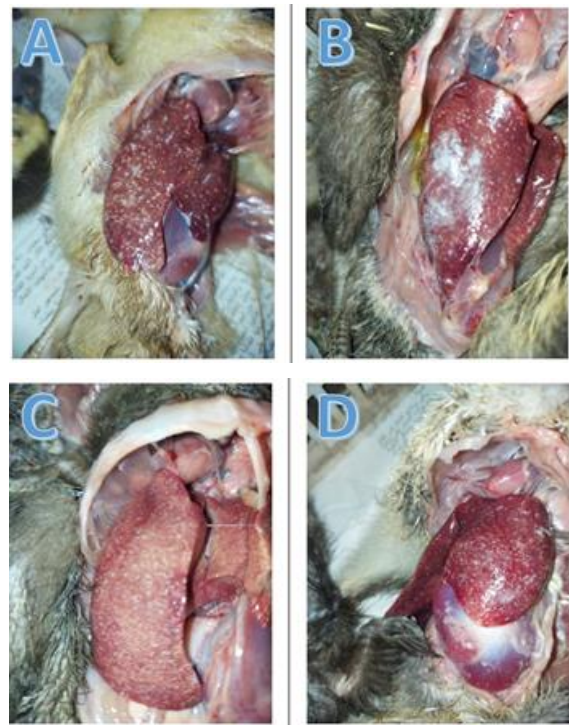


Fig. 1: PM lesions in G2 (control positive) showed severe cheesy deposit of fibrinous exudate on the pericardium, liver capsules and air sacs as revealed in A,B,C and D.

4. DISCUSSION

Riemerella anatipestifer infections continue to cause substantial economic losses in the duck production industry, necessitating improved disease control strategies. The presence of numerous serotypes, a lack of cross-protection, and varying field outbreak serotypes present significant challenges in developing an effective vaccine (Shunzhou et al., 2002). Past methods, which rely on autologous bacterins, have provided limited protection against heterologous strains (Pathanasophon et al., 1996). Formalin-killed *R. anatipestifer* vaccines have shown promise in inducing both humoral and cellular immunity, yet their effectiveness against different serotypes remains limited (Hu et al., 2001; Huang et al., 2002; Liu et al., 2013; Shunzhou et al., 2002)

4.2. Vaccine Formulation and Immunogenic Response

This study focused on developing three *R. anatipestifer* formalin-killed vaccines containing prevalent serotypes 1 and 2 adjuvanted with montanide ISA70, paraffin oil, and S. Typhimurium LPS. Monitoring the cellular immune response using nitric oxide concentration and evaluating humoral immunity through IHA and ELISA revealed distinct differences among the vaccinated groups. The group receiving the *R. anatipestifer* vaccine adjuvanted with S. Typhimurium LPS exhibited the highest immune response and protection percentage, in line with previous studies highlighting the efficacy of such adjuvants in improving vaccine efficacy (De Boever et al., 2008; Khatsenko et al., 1993; Son and Kim, 1995; Sunwoo et al., 1996)

Analysis post-challenge with serotypes 1 and 2 underscored those ducks in Group 5, vaccinated with S. Typhimurium LPS-adjuvanted vaccine, and displayed significantly higher nitric oxide levels, indicating robust cellular immune responses. Moreover, the same group exhibited elevated antibody titers as assessed by IHA and ELISA tests, correlating with enhanced protection percentages against both serotypes. Ducks in Groups 3 and 4 showed intermediate responses, with Group 3 demonstrating higher

immune responses and protection than Group 4. These results are in accordance with previous researchers who declared the robust effect of *S. Typhimurium* LPS-adjuvant on both cellular and humoral immune response (De Boever et al., 2008; Khatsenko et al., 1993; Son and Kim, 1995; Sunwoo et al., 1996)

Clinical observations highlighted stark differences between the control positive group, exhibiting severe clinical signs and postmortem lesions, and vaccinated groups, which displayed minimal symptoms and lesions. The findings emphasize the potential of the *S. Typhimurium* LPS-adjuvanted vaccine in mitigating disease severity

Moving forward, future research should explore further refinements in vaccine formulations, potentially incorporating additional prevalent serotypes. Explorations into other novel adjuvants to enhance cross-protection and immunogenicity against a broader spectrum of *R. anatipestifer* serotypes are warranted.

5. CONCLUSIONS

The study demonstrates that the developed *R. anatipestifer*-killed vaccine adjuvanted with *S. Typhimurium* LPS effectively induces both cellular and humoral immune responses, leading to robust protection against prevalent serotypes 1 and 2. The outcomes suggest promising avenues for enhancing disease control measures in duck populations and advocate for the potential of tailored vaccines in combatting *R. anatipestifer* infections.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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