

Field Assessment of the Level of Protection Conferred by a Newly Prepared Combined Inactivated Vaccine against *E. coli* and *P. multocida* in Rabbit in Egypt

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Abstract

Pasteurellosis is the most prevalent, extremely contagious bacterial disease among domestic rabbits and is considered the leading cause of deaths in rabbits, resulting in enormous economic losses to the rabbit industry. Screening for bacterial agents causing mortalities in rabbits revealed the presence of Enterobacteriaceae species in approximately 42% of studied cases, with *E. coli* the most commonly isolated organism. The present study was designed to evaluate the immune response of rabbits vaccinated with a locally prepared, combined inactivated vaccine of *Pasteurella multocida* and *E. coli*, adjuvanted with Montanide ISA70. A total of 370 rabbits, aged 2 - 3 weeks, were divided into four groups: (G1) vaccinated with a polyvalent *P. multocida* vaccine, (G2) vaccinated with a polyvalent *E. coli* vaccine, (G3) vaccinated with a combined inactivated Montanide ISA70 vaccine of *P. multocida* and *E. coli*, and (G4) kept as a non-vaccinated control group. All rabbits received two doses of 0.5 ml of the prepared vaccines, administered one month apart, and were then challenged with virulent strains of *P. multocida* and *E. coli* three weeks after the second vaccination. The prepared vaccines were evaluated by determining humoral immunity using indirect haemagglutination (IHA) test and ELISA. The potency of the vaccines was assessed through challenge and determination of LD50. Experimental findings on the prepared polyvalent combined inactivated *P. multocida* and *E. coli* vaccine indicated that it is a potent vaccine, producing the highest antibody titers and a 90% protection rate against challenges with virulent strains of *P. multocida* type A, D2, and *E. coli* types O157, O151 and O125. Thus, this vaccine is promising in addressing both *P.*

multocida and *E. coli* problems in rabbits, farms, providing significant protection, and we recommend its commercial production to help rabbit producers control these two major bacterial infections.

Keywords

P. multocida, *E. coli*, Vaccine, ELISA, Challenge Test, Rabbit Protection

1. Introduction

One of the world's most pressing concerns is how to eradicate poverty and feed the growing population with a protein-rich, cholesterol-free diet to prevent malnutrition. Rabbits provide a practical solution to these issues [1]. Therefore, rabbits are among the most economically important small livestock animals, and their protection and disease control are very crucial [2]. Pasteurellosis and colibacillosis are contagious bacterial diseases in domestic rabbits. Pasteurellosis can infect rabbits immediately after birth, and its prevalence can reach more than 90% by the age of five months [3], causing enormous economic losses to the rabbit industry [4]. *P. multocida* causes snuffle, a highly contagious disease that primarily affects upper respiratory tracts of rabbits and can be fatal. It can lead to abscesses, septicemia, pyometritis, conjunctivitis, otitis media, and enzootic pneumonia [5]. *P. multocida*, the causative agent of Pasteurellosis, is categorized into five capsular serogroups: A, B, D, E, and F. Serotypes A and D are commonly associated with rabbit pasteurellosis [4]. *E. coli*, the causative agent of colibacillosis, is linked to the development of digestive diseases in rabbits and remains one of the leading causes of economic losses. Screening for bacterial agents causing mortalities in rabbits identified Enterobacteriaceae species in approximately 42% of studied cases, with *E. coli* being the most commonly isolated organism (24.29%) [6]. Rabbits can contract two forms of colibacillosis, depending on their age. Rabbits aged 1 - 2 weeks may suffer from severe yellowish diarrhea with a high mortality rate, while the enterotoxemia form occurs in weaned rabbits aged 4 - 6 weeks, presenting as petechial hemorrhages on the intestinal serosa [7]. The prevalent serogroups of virulent *E. coli* in rabbits are O153 (28%), O27 (24%), untypable (20%), O125 (24%), and O158 (12%) [8]. *E. coli* O157 is the prototypical enterohaemorrhagic strain of *E. coli* that produces Shiga toxin and is responsible for food and waterborne outbreaks, as well as severe bloody diarrhea [9]. The purpose of this study was to create and evaluate a locally prepared, combined inactivated *P. multocida* and *E. coli* vaccine with Montanide ISA70 adjuvant for use in rabbits, as pasteurellosis and *E. coli* infections significantly contribute to morbidity and mortality, leading to high economic losses in commercial rabbit production.

2. Material and Methods

2.1. Bacterial Strains

P. multocida strains A1, A3, A12, and D2, along with *E. coli* serotypes O125, O151,

and O157 were used in the preparation of inactivated polyvalent *P. multocida*, inactivated polyvalent *E. coli*, and combined inactivated *P. multocida* and *E. coli* vaccines, as well as challenge test. The strains were supplied by the Aerobic Bacterial Vaccines Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo.

2.2. Experimental Animals

2.2.1. White Swiss Mice

A total of 170 Swiss white mice, weighing 25 - 30 g, were used. Twenty mice were used to assess the safety of the produced vaccinates, while the remaining 150 mice were used to determine the LD50 for both the prepared inactivated *P. multocida* vaccine and the combined inactivated *P. multocida* and *E. coli* vaccine.

2.2.2. Rabbits

A total of 380 *P. multocida* seronegative rabbits, about 2 - 3 weeks of age and weighing 1.5 - 2 Kg, were used. Ten rabbits were used for the passage of *P. multocida* and *E. coli* strains while the remaining 370 rabbits were used for evaluation of prepared vaccines. The rabbits had not been previously vaccinated or treated with antibiotics and were reared in accordance with biosafety and biosecurity guidelines.

2.3. Adjuvant

Montanide ISA 70 (Seppic, Paris, France) is an oil/surfactant-based adjuvant. It was developed for the production of water-in-oil (W/O) emulsions.

2.4. Vaccines Preparation [10] [11]

2.4.1. Preparation of the Inactivated *P. multocida* Antigenic Phase

P. multocida strains A1, A3, A12, and D2 were grown separately in tryptic soy broth (TSB) for 24 hours at 37°C with continuous shaking. Each strain was standardized at 4×10^9 CFU/ml. The bacteria were inactivated using 0.5% formalin (37% - 41% concentration) and cultured for 24 hours at 37°C. After complete inactivation, each culture was assessed for purity, safety, and sterility according to [12] guidelines. Cultures were then mixed evenly, preserved with 0.01% thiomersal, and stored at 4°C until needed.

2.4.2. Preparation of the Inactivated *E. coli* Antigenic Phase

E. coli serotypes O157, O151, and O125 were cultured in brain heart infusion broth and adjusted to a concentration of 1.2×10^8 CFU/ml. The cultures were then inactivated using 0.5% formalin (37% - 41% concentration) and incubated at 37°C for 24 hours. After complete inactivation, each culture was assessed for purity, safety, and sterility according to [12] guidelines. The cultures were combined in equal amounts, preserved with 0.01% thiomersal, and stored at 4°C until needed.

2.4.3. Preparation of the Inactivated *P. multocida*, Inactivated *E. coli*, and Combined Inactivated *P. multocida* and *E. coli* Vaccine Formulations

Inactivated *P. multocida*, inactivated *E. coli*, and combined inactivated *P.*

multocida and *E. coli* vaccines were prepared using the formulation method recommended by Montanide™ manufacturer. Montanide™ ISA 70 was used to create a water-in-oil (W/O) emulsion vaccine with a 30/70 (W/W) aqueous/oil ratio. For the combined inactivated *P. multocida* and *E. coli* vaccine, equal amounts of *P. multocida* and *E. coli* antigens were added to ensure that the antigenic content for both antigens was at least 10⁸ EID₅₀. Stable preparations were achieved by combining the aqueous medium with Montanide™ ISA 70 at room temperature or lower and vigorously stirring for 15 - 30 minutes, as advised by the manufacturer's instructions.

2.5. Quality Control of Vaccine Preparation

The prepared vaccines were tested for sterility, safety, and purity using the established worldwide standards outlined [13].

2.6. Evaluation of the Prepared Vaccines

2.6.1. Determination of the Lethal Dose Fifty (LD₅₀) in Mice

The potency of the produced inactivated *P. multocida* and combined inactivated *P. multocida* and *E. coli* vaccines was initially assessed by determining the LD₅₀ in mice, as outlined by [12]. The mice were divided into three groups: the first group (50 mice) was vaccinated with the prepared inactivated *P. multocida* vaccine, the second group (50 mice) was vaccinated with the combined inactivated *P. multocida* and *E. coli* vaccine, and the third group (50 mice) served as an unvaccinated control. The vaccinated groups received a booster dose three weeks later and were subsequently challenged three weeks after the booster to determine the lethal dose fifty (LD₅₀). The vaccinated mice were separated into 10 groups and challenged with a tenfold serial dilution of mixed virulent *P. multocida* strains (5 vaccinated and 5 unvaccinated animals per dilution). Mortality rates were monitored and recorded for one week. Reference [14] formula was used to compute the lethal dose (LD₅₀).

$$\text{LD } 50\% = \text{above } 50 - 50 / \text{above } 50 - \text{below } 50$$

Depending on the satisfactory results of the protective value of the prepared vaccines in mice, the experimental design was then applied to the target animal, rabbits.

2.6.2. Experimental Design

Out of three hundred and seventy rabbits aged 2 - 3 weeks, all were reared under proper management conditions, including appropriate food, biosafety and biosecurity measures with no history of previous infection or vaccination. The rabbits were divided into four groups for the experiment. Group 1 (85 rabbits) was vaccinated with the polyvalent inactivated *P. multocida* vaccine, Group 2 (65 rabbits) was vaccinated with the polyvalent inactivated *E. coli* vaccine, and Group 3 (145 rabbits) received the combined inactivated polyvalent *P. multocida* and *E. coli* vaccine. Group 4 (75 rabbits) was maintained as a non-vaccinated control group

Table 1.

Table 1. Experimental design.

Vaccines	<i>P. multocida</i> vaccine (G1)	<i>E. coli</i> vaccine (G2)	Combined <i>P. multocida</i> and <i>E. coli</i> (G3)	Control (G4)
No. of lab animal	85 rabbits	65 rabbits	145 rabbits	75 rabbits
Depending on the type of vaccine, all groups received the initial dose followed by a booster dose four weeks later.				
Challenge 3 weeks post boosting	80 rabbits were challenged with <i>P. multocida</i> strains (20 rabbits/strain)	60 rabbits were challenged with strains of <i>E. coli</i> (20 rabbits/strain)	80 rabbits were challenged with <i>P. multocida</i> strains and 60 rabbits were challenged with strains of <i>E. coli</i> (20 rabbits/strain)	40 rabbits were challenged with <i>P. multocida</i> strains and 30 rabbits were challenged with strains of <i>E. coli</i> (10 rabbits/strain)
Five rabbits from each group were kept without challenge for blood sampling to follow up the immune response duration of each vaccine				

2.6.3. Vaccine Inoculation

All rabbit groups received two doses of 0.5 ml subcutaneously of the prepared vaccines, with one-month interval between doses. Rabbits in all groups (except for 5 rabbits from each group, which were kept for blood sample collection) were challenged with virulent strains of *P. multocida* or *E. coli* depending on the vaccine type, three weeks after booster vaccination.

2.6.4. Blood Sample Collection

Random blood samples were collected every two weeks post-vaccination and the humoral immune response was evaluated serologically.

2.6.5. Evaluation of the Humoral Immune Response

1) Indirect haemagglutination test (IHA)

The IHA test was used to assess the humoral immune response to *P. multocida*. The glutaraldehyde treated RBCs and capsular antigens of *P. multocida* strains were produced in accordance with [15]. The vaccine was considered effective if it induced seroconversion in the sera of vaccinated rabbits.

2) Enzyme linked immunosorbent assay (ELISA)

The antibody titers against *P. multocida* and *E. coli* in the serum of vaccinated rabbits were determined using the ELISA assay described by [16].

2.6.6. Challenge Test

Rabbits vaccinated with the inactivated *P. multocida* and the combined inactivated *P. multocida* and *E. coli* vaccines (Groups 1 and 3), along with the control group, were challenged with 0.1ml of $1 - 2 \times 10^2$ CFU of *P. multocida* strains A1, A3, A12, and D2 (20 vaccinated and 10 control rabbits per strain). Similarly, rabbits vaccinated with the inactivated *E. coli* and combined inactivated *P. multocida* and *E. coli* vaccines (Groups 2 and 3), along with the control group, were

challenged with 0.1 ml of 1.2×10^6 CFU of *E. coli* strains O157, O151, and O125 (20 vaccinated and 10 control rabbits per strain) to estimate the protection percentage of the prepared vaccines. Following the challenge, rabbits were monitored daily for a week for any mortality or clinical symptoms. The clinical findings of both vaccinated and control rabbits were evaluated and documented.

2.6.7. Statistical Analysis

The statistical analysis of the obtained data was performed using SPSS software, version 21 (IBM Corp., Armonk, NY, USA, 2012). A t-test was employed to compare the means between groups and assess the significance of differences in the outcomes of interest. The data were first checked for normality using the Shapiro-Wilk test to ensure that they met the assumptions of the t-test. A two-sample independent t-test was used to compare the differences between the vaccinated groups in terms of humoral immune response. For comparisons between groups, a paired t-test was applied. The level of significance was set at $p < 0.05$. Results were presented as mean \pm standard deviation (SD), and 95% confidence intervals (CIs) were calculated to interpret the magnitude of the effects.

2.6.8. Ethical Consideration

The project was approved by the Institutional Animal Care and Use Committee (ARC-IACUC) of the Agricultural Research Center, under the approval code ARC CLEVB 122-24.

3. Results

3.1. Results of Quality Control of Prepared Vaccines

3.1.1. Purity Test

The cultures of *P. multocida* and *E. coli* used in prepared vaccines were confirmed to be pure.

3.1.2. Sterility Test

The prepared vaccines were free from bacterial, fungal, and Mycoplasma contamination as described by [12].

3.1.3. Safety Test

The prepared vaccines were found to be safe, producing no clinical, local, systemic or necropsy findings during a two-week observation period after injection of a double field dose of each vaccine.

3.2. Results of Lethal Dose Fifty (LD50) in Mice

The potency of the prepared inactivated *P. multocida* and the combined inactivated *P. multocida* and *E. coli* vaccines was initially evaluated by determining the LD50 in mice. The vaccinated mice exhibited LD50 values that were 2.00 and 2.12 log units higher than those of the control mice for the single and combined vaccines, respectively.

3.3. Result of Indirect Haemagglutination Test (IHA) against *P. multocida* Strains in Vaccinated Rabbits

According to **Table 2**, the IHA test on serum samples from rabbits vaccinated with inactivated *P. multocida* and the combined inactivated *P. multocida* and *E. coli* vaccines demonstrated detectable *P. multocida* antibodies as early as the second week after vaccination. The antibody titers reached 32, 64, 32, and 16 for strains A1, A3, A12, and D2, respectively, in the inactivated *P. multocida* vaccine, while they reached 64, 64, 64, and 32 for the same strains in the combined inactivated *P. multocida* and *E. coli* vaccine. By the 4th week post vaccination, the antibody titers increased to 64, 128, 64, and 32 for strains A1, A3, A12, and D2, respectively, in the inactivated *P. multocida* vaccine, while they increased to 128, 128, 128, and 64 for the same strains in the combined inactivated vaccine. The booster dose administered four weeks after the primary vaccination led to a rapid increase in antibody titers, peaking at the 4th week post-boost and persisting until the 6th week, after which they decreased at the 8th post-boost as shown in **Table 2**. Data from this test indicate that the combined inactivated *P. multocida* and *E. coli* vaccine showed slightly higher antibody titers compared to the inactivated *P. multocida* vaccine alone. Statistical analysis of the data revealed a p value of 0.014 between the single and combined vaccines, indicating a statistically significant difference.

Table 2. Antibody titers in the sera of rabbits vaccinated with the inactivated *P. multocida* vaccine (Group 1), the combined inactivated *P. multocida* and *E. coli* vaccine (Group 3), and control group (Group 4) as determined by IHA test.

Sampling date	Vaccinated groups								(G4)
	(G3)				(G1)				
	A1	A3	A12	D2	A1	A3	A12	D2	
Pre-vaccination	2	2	2	4	2	4	2	2	0
First vaccination									
2 weeks PV	32	64	32	16	64	64	64	32	2
4 weeks PV	64	128	64	32	128	128	128	64	0
Second vaccination (Booster Dose)									
2 weeks PB	128	256	128	64	256	512	256	128	2
4 weeks PB	256	512	256	128	512	1024	512	256	0
6 weeks PB	256	512	256	128	512	1024	512	256	2
8 weeks PB	128	256	128	64	256	512	256	128	0

PV: post vaccination; PB: post boosting.

3.4. Results of ELISA against *P. multocida* Strains in Vaccinated Rabbits

Table 3 shows that the ELISA conducted on serum samples from rabbits vaccinated with the inactivated *P. multocida* and the combined inactivated *P.*

multocida and *E. coli* vaccines revealed detectable *P. multocida* antibodies starting from the second week post-vaccination. The antibody titers reached 1279, 1078, 1688, and 965 for strains A1, A3, A12, and D2, respectively, in the inactivated *P. multocida* vaccine, while reached 1876, 1190, 1876, and 1039 for the same strains in the combined vaccine. At the 4th week post-vaccination, the antibody titers increased to 1714, 1636, 2254, and 1636 for strains A1, A3, A12, and D2, respectively, in the inactivated *P. multocida* vaccine, and to 1995, 2599, 2487, and 2166 for the same strains in the combined vaccine. Following the booster dose, the antibody titers in the inactivated *P. multocida* vaccine rapidly increased four weeks after the primary vaccination, peaking at 3164, 3286, 3254, and 2927 for A1, A3, A12, and D2, respectively. In the combined vaccine, the titers peaked at 3256, 3455, 3490, and 3288 for the same strains and persisted through the 6th week. By the 8th week post-booster, the titers had decreased to 1279, 1186, 1433, and 1487 for A1, A3, A12, and D2, respectively, in the inactivated *P. multocida* vaccine, and to 1354, 1251, 2250, and 2318 in the combined vaccine. Data from this test indicate that the combined inactivated *P. multocida* and *E. coli* vaccine produced slightly higher antibody titers compared to the inactivated *P. multocida* vaccine alone. Statistical analysis of the data revealed a p-value of 0.015 between the single and combined vaccines, indicating a statistically significant difference.

Table 3. Antibody titers against *P. multocida* strains in the sera of rabbits vaccinated with the inactivated *P. multocida* vaccine (Group 1), the combined inactivated *P. multocida* and *E. coli* vaccine (Group 3), and control group (Group 4) as determined by ELISA.

Sampling date	Vaccinated groups								(G4)
	(G1)				(G3)				
	A1	A3	A12	A3	A1	D2	A12	A3	
Pre-vaccination	0	0	120	0	0	0	0	0	120
First vaccination									
2 weeks PV	1279	1078	1688	965	1876	1190	1876	1039	140
4 weeks PV	1714	1636	2254	1636	1995	2599	2487	2166	196
Second vaccination (Booster Dose)									
2 weeks PB	2423	2356	2966	2265	2517	2455	3110	2920	184
4 weeks PB	3164	3286	3254	2927	3256	3455	3490	3288	84
6 weeks PB	3180	3290	3287	2987	3288	3480	3510	3320	109
8 weeks PB	1279	1186	1433	1487	1354	1251	2250	2318	13

PV: post vaccination; PB: post booster.

3.5. Results of ELISA against *E. coli* Strains in Vaccinated Rabbits

As indicated in **Table 4**, the ELISA conducted on serum samples from rabbits vaccinated with the inactivated *E. coli* and the combined inactivated *P. multocida* and *E. coli* vaccines showed detectable *E. coli* antibodies as early as the second week after vaccination. The antibody titers reached 1612, 1587, and 1574 for

strains O151, O157, and O125, respectively, in the inactivated polyvalent *E. coli* vaccine, while they reached 2013, 1938, and 1987 for the same strains in the combined vaccine. At the 4th week post-vaccination, the antibody titers increased to 2809, 2789, and 2768 for strains O151, O157, and O125, respectively, in the inactivated polyvalent *E. coli* vaccine, and to 2980, 2940, and 2801 for the same strains in the combined vaccine. Booster vaccination administered four weeks after the primary vaccination caused a rapid rise in antibody titers, which peaked at the 4th week post-booster, as detailed in **Table 4**. These antibody levels persisted through the 6th week but then decreased gradually by the 8th week post-booster. The data indicate that the combined inactivated *P. multocida* and *E. coli* vaccine elicited slightly higher antibody titers compared to the inactivated polyvalent *E. coli* vaccine. Statistical analysis showed a p-value of 0.047 between the single and combined vaccines, indicating a statistically significant difference.

Table 4. Antibody titers against *E. coli* in the sera of rabbits vaccinated with the inactivated *E. coli* vaccine (Group 2), the combined inactivated *P. multocida* and *E. coli* vaccine (Group 3), and control group (Group 4) as determined by ELISA.

Sampling date	Vaccinated groups						Control (G4)
	Inactivated <i>E. coli</i> vaccine (G2)			Combined inactivated vaccine (G3)			
	O151	O157	O125	O151	O157	O125	
Pre-vaccination	0	0	0	0	0	0	0
First vaccination							
2 weeks PV	1612	1587	1574	2013	1938	1987	93
4 weeks PV	2809	2789	2768	2980	2940	2801	70
Second vaccination (Booster Dose)							
2 weeks PB	4670	4588	4554	4777	4743	4633	127
4 weeks PB	6690	6591	6543	7160	7092	6821	206
6 weeks PB	6630	6551	6488	7092	6933	6780	133
8 weeks PB	5598	5451	5291	5692	5592	5451	140

PV: post vaccination; PB: post boosting.

3.6. Results of Challenge Test

In the vaccinated rabbits, the protection rates observed in the challenge test with *P. multocida* strains (A1, A3, A12, and D2) showed the following results: for rabbits receiving the inactivated *P. multocida* vaccine (Group 1), the protection percentages were 80%, 85%, 85%, and 90% for strains A1, A3, A12, and D2, respectively. For rabbits receiving the combined inactivated *P. multocida* and *E. coli* vaccine (Group 3), the protection percentages were 85%, 90%, 90%, and 95% for the same strains. In contrast, control rabbits exhibited protection rates of 20%, 10%, 20%, and 20% for the respective strains, as shown in **Table 5**. Conversely, the protection rates from the challenge test with *E. coli* strains (O151, O157, and

O125) are presented in **Table 6**. For rabbits receiving the inactivated *E. coli* vaccine (Group 2), the protection percentages were 80%, 80%, 85%, and 90% respectively, and 85%, 90%, and 95% for the same strains in the combined vaccine. The control rabbits exhibited protection rates of 20% for all three strains.

Table 5. Mean protective efficacy against *P. multocida* strains in rabbits vaccinated with inactivated *P. multocida* and combined inactivated *P. multocida* and *E. coli* vaccines.

Type of vaccine	Inactivated <i>P. multocida</i> vaccine				Combined inactivated vaccine				Control			
	(G1)				(G3)				(G4)			
	A1	A3	A12	D2	A1	A3	A12	D2	A1	A3	A12	D2
Total no. of rabbits	20	20	20	20	20	20	20	20	10	10	10	10
Dead	4	3	3	2	3	2	2	1	8	9	8	8
Survived	16	17	17	18	17	18	18	19	2	1	2	2
Protection %	80%	85%	85%	90%	85%	90%	90%	95%	20%	10%	20%	20%

Table 6. Mean protective efficacy against *E. coli* strains in rabbits vaccinated with inactivated *E. coli* and combined inactivated *P. multocida* and *E. coli* vaccines.

Type of vaccine	<i>E. coli</i> vaccine			Combined inactivated vaccine (G3)			Control		
	(G2)			(G3)			(G4)		
	O151	O157	O125	O151	O157	O157	O151	O157	O125
Total no. of rabbits	20	20	20	20	20	20	10	10	10
Dead	4	3	2	3	2	2	8	8	8
Survived	16	17	18	17	18	18	2	2	2
Protection %	80%	85%	90%	85%	90%	90%	20%	20%	20%

4. Discussion

Raising rabbits can boost revenue and is regarded as a high-quality source of animal protein with great biological value for people with limited resources in Egypt [11]. Rabbits, like other animal species, can contract a range of illnesses and infestations, resulting in varying degrees of economic loss [17]. *P. multocida* is the most devastating pathogen, resulting in enormous economic losses in rabbit production [18]. Based on lipopolysaccharide (LPS), which is a key immunogen, influencing the vaccine's ability to protect against infection and a critical virulence factor, *P. multocida* is divided into 16 serotypes [19]. Conversely, *E. coli* is typically absent from rabbits' intestinal flora. Nevertheless, it can be a significant factor in both enteritis and death, particularly in suckling rabbits. Non-toxin producing, enteropathogenic strains of *E. coli* can cause severe illness in rabbits. It is important for rabbit owners to be aware of the potential risks associated with *E. coli* infection and take appropriate precautions to prevent them. The severity of

E. coli strains varies, and various strains have been discovered in rabbit enteritis epidemics. Eliminating *E. coli* infection is challenging due to about 42% of digestive disorders attributed to antibiotics treatment and resistance [6]. With the use of vaccinations, some diseases have been eradicated and the frequency of many infectious diseases has decreased [3]. This study aimed to develop a combined inactivated vaccine adjuvanted with Montanide ISA70 to address both *P. multocida* and *E. coli*. The potency of the prepared vaccine was assessed through seroevaluation using the IHA test and ELISA, as well as a challenge test and determination of LD50, in comparison with prepared inactivated single *P. multocida* and *E. coli* vaccines. The protective value of the prepared inactivated *P. multocida* and the combined inactivated *P. multocida* and *E. coli* vaccines was initially evaluated by determining the protection log in mice (LD50). The minimal level of protection is a two-log difference between vaccinated and unvaccinated control mice after a booster dose [20]. The study's findings revealed that the vaccinated mice had 2.00 and 2.12 logs, higher protective values than control mice for the inactivated single and combined vaccines, respectively. Reference [21] obtained similar results by comparing single-dose evaluations of rabbit Pasteurellosis vaccines with booster dose evaluations in mice. Reference [22] observed that the inactivated rabbit pasteurellosis vaccine adjuvanted with Montanide ISA 70 induced 3.85 and 3.69 logs of protection in mice against *P. multocida* type A and D2, respectively. Based on the satisfactory results of the protective value of the prepared vaccines in mice, the experimental design was planned for target animals (rabbits), as showed in **Table 1**. The humoral immune response was determined by the IHA test for *P. multocida* **Table 2** and measured by ELISA for *P. multocida* and *E. coli* (**Tables 3-4**). These tests demonstrated an early and strong immune response starting from the second week post-vaccination. Inoculation of a booster dose 4 weeks after the primary vaccination rapidly increased antibody titers, peaking at the 4th week post-booster and continuing until the 6th week post-booster before decreasing by the 8th week post-booster. Furthermore, the combined inactivated *P. multocida* and *E. coli* vaccine produced significantly higher levels of antibody titers than the inactivated *P. multocida* and inactivated *E. coli* vaccines alone. These findings are consistent with [22], who found that inactivated rabbit pasteurellosis vaccines adjuvanted with Montanide ISA 70 generated protective antibodies against *P. multocida* and provided high and long-lasting antibodies responses, as evaluated by IHA. Additionally, [23] reported that the hemorrhagic septicemia oil adjuvant vaccine (HSOAV) provided protective antibody titers up to 300 days post-booster, with antibodies detectable in the serum of vaccinated animals for up to 420 days. Evaluation of the protection rates obtained in this study using the challenge test with *P. multocida* strains (A1, A3, A12, and D2) showed that rabbits receiving the inactivated *P. multocida* vaccine (Group 1) had protection percentages of 80%, 85%, 85%, and 90% for strains A1, A3, A12, and D2, respectively. In comparison, rabbits receiving the combined inactivated *P. multocida* and *E. coli* vaccine (Group 3) had protection percentages of 85%, 90%, 90%, and 95%, while the control rabbits had protection rates of 20%, 10%, 20%,

and 20%, as shown in **Table 5**. In the same context, [22] demonstrated that Montanide ISA50-adjuvanted rabbit pasteurellosis vaccination provided 100% and 90% protection, while mineral oil provided 80% and 80% protection against serotypes A and D, respectively. Additionally, [24] confirmed that Montanide ISA50-adjuvanted vaccines induced early and long-lasting antibodies with high protection percentages compared with paraffin oil adjuvants. Regarding the protection rates using the challenge test with *E. coli* strains (O151, O157, and O125), rabbits receiving the inactivated *E. coli* vaccine (Group 2) had protection percentages of 80%, 85%, and 90%, respectively, while those receiving the combined inactivated *P. multocida* and *E. coli* vaccine (Group 3) had protection percentages of 85%, 90%, and 95%. Control rabbits showed 20% protection for all strains, as shown in **Table 6**. Reference [25] reported similar findings, stating that significant outer membrane components of gram-negative bacteria have been identified as inhibitors of infection in mammals and fish. According to [26], immunization of chickens with entire cells of *E. coli* induces high levels of anti-LPS activity, suggesting that LPS is a potent antigen in chickens. From these data, it was clear that the antibody titers measured with IHA and ELISA tests were highly correlated with protection against challenge with virulent organisms.

5. Conclusion

In conclusion, a single shot of an oil-based vaccine may confer an immune response, but a booster shot provides the maximum protective immune response for a longer duration. Additionally, the combination of *P. multocida* and *E. coli* increased antibody titers and protection percentages compared to single vaccines. Therefore, the combined vaccine is recommended not only to reduce the cost of producing two single vaccines or decrease the stress of two injections but also for its significantly higher immune response against both diseases. It is promising for protection against both pasteurellosis and colibacillosis in rabbits and will facilitate overall sanitary and medical prevention against the main epidemiologically relevant strains of both pathogens. A potent vaccine containing outer membrane proteins and lipopolysaccharides from various strains of *P. multocida* (A1, A3, A12, and D2) and *E. coli* (O151, O157, and O125) is expected to protect rabbits against field infections of pasteurellosis and colibacillosis. Future studies will explore the field application of this vaccine on rabbit farms across different Egyptian governorates.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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