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Preparation and evaluation of a novel combined inactivated vaccine against Pasteurellosis and *E. coli* infection in Sheep.

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ABSTRACT

Pneumonic Pasteurellosis is a common disease in sheep that affects the sheep industry. Also, *E. coli*, which affects young lambs and adult sheep, causing morbidity and mortality, threatens sheep's wealth. Therefore, an inactivated adjuvanted combined vaccine against pasteurellosis and *E. coli* infection was prepared to counteract their catastrophic effects. In this study sheep aged 6 weeks were divided into four groups. The first group was immunized with *P. multocida* (A, D, B6) and *M. haemolytica* (A, *P. trehalosi* type T) vaccine. The second was immunized with the *E. coli* (K99) vaccine. The third was immunized with a combined *P. multocida*, *M. haemolytica*, and *E. coli* vaccine. The fourth was kept as a non-vaccinated control group. All sheep were injected subcutaneously with two doses (2 weeks apart) of the vaccines (1 ml/dose). The vaccination of sheep was at six weeks of age. The highest antibody titers after boostering for groups vaccinated with *P. multocida* and *M. haemolytica* vaccines were as follows: the 1st group and the 3rd group for type A, B6, and *P. trehalosi* at the 4th month, but *P. multocida* type D at the 5th month and *M. haemolytica* type A at the 3rd month. The 2nd and 3rd groups had the highest antibody titers for *E. coli* at the 3rd month. Statistically, there were no significant differences between the results of the combined or single vaccines. Briefly, the combined inactivated vaccine against pasteurellosis and *E. coli* has good effects, with priority given to protecting sheep against these diseases and decreasing the stress on the livestock and the efforts of vaccination.

1. INTRODUCTION

One of the most common diseases in sheep is pneumonia, which has an adverse effect on sheep investment globally. The disease was commonly noticed to occur after stress factors, either physical or physiological. Viral and bacterial infections also predispose to pneumonia (Brogden et al., 1998). Pneumonic Pasteurellosis is one of the critical diseases that need control. Vaccination is the most applicable method with good economic feedback (Marru et al., 2013). Respiratory Mannheimiosis is a synonym for pneumonic Pasteurellosis, which is widely spread in ruminants. That disease is infectious and fatal, leading to adverse economic mortality that can reach 30% of deaths worldwide (Tewodros and Annania, 2016). *Pasteurella and Mannheimia* are gram-negative facultative anaerobic bipolar Coccobacilli nonmotile bacteria (Abd El-Moneim et al., 2022). *Escherichia coli (E. coli)* is a gram-negative bacterium frequently isolated from clinical cases of acute mastitis (Klaas et al., 2018; Nagasawa et al., 2019). Young lambs and adult sheep are particularly susceptible to the morbidity and mortality caused by colibacillosis (Munoz et al., 1996). Neonatal and infant mortality in small ruminants is a serious problem that costs the country financially and reduces the productivity and profits of farmers. Enteropathogenic strains of *E. coli* colonize the small intestine and produce enterotoxins, resulting in the loss of fluid and electrolytes. Such a loss of body fluid, causes severe diarrhea leading to dehydration, electrolyte imbalance, and metabolic acidosis (Wary and Thomlinson, 1975; Yano et al., 1995). Most of these enteropathogenic *E. coli* (ETEC) strains share a common surface antigen (K99). The antigen exists in the form of pili or hair-like droplet projections that cause the body to attach to the intestinal tract, promoting colonization and causing pathological effects (Horiet et al., 1989; Cameron and Fuls, 1970). In the North of Upper Egypt and by using PCR tests for *P. multocida* and *M. haemolytica* investigation in ruminants, the results showed that 87.9% for *P. multocida* and 100% for *M. haemolytica* isolates were positive (Abed et al., 2020). *M. haemolytica* was isolated from sheep in abattoirs in Egypt at a prevalence rate of 14.10% (Kaoud et al., 2010). In an investigation of the epidemiological study of *P. multocida* in sheep infections in Egypt, the occurrence of *P. multocida* was 13.75% (Bahr et al., 2021). For determining the occurrence of *E. coli* K99 in Egypt, rectal swabs from

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diarrheic calves, sheep, and goats were taken and investigated. The *E. coli* prevalence rate was 63.6% in calves, 27.3% in goat and 9.1% in sheep (Osman et al., 2013). The colibacillosis prevalence in Egypt differs from 5.4 to 100%, and it is roughly estimated the mortality related to colibacillosis of 20% may reduce net profit to 40% (Tarabees et al., 2021). Therefore, vaccination could result in successful disease control (Rahman et al., 2016). The use of a combined vaccine against infectious diseases that revealed protective immunity, as well as a single vaccine, was recommended by many authors (Hanna et al., 2014). This study aimed to prepare a combined inactivated vaccine against Pasteurellosis and *E. coli* in sheep and evaluate the immune response to determine the vaccine efficacy in sheep against both diseases.

2. MATERIAL AND METHODS

2.1. Ethical and study protocol approval:

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

2.2. Vaccine preparation Strains:

P. multocida types A, D, B6, *M. haemolytica* type A, *P. trehalosi* type T, and *E. coli* K99 were obtained kindly from the Aerobic Bacteria Research Department, VSVRI, ARC, Egypt.

2.3. Adjuvant:

Montanide™ ISA 206 VG (SEPPIC Co., France) was used as an adjuvant for the preparation of the vaccines.

2.4. P. multocida and M. haemolytica Antigenic Culture preparation (Rahman et al., 2016):

The *Pasteurella* strains were inoculated separately in nutrient broth enriched with yeast extract. The inoculating media were incubated at 37 °C for 24 hr. in a bacteriological incubator for bacterial growth. Then inactivation with formalin. The inactivated cultures of *P. multocida* biotypes A, D, and B6 were adjusted to contain 1×10^7 CFU for each, and those of *M. haemolytica* type A and *P. trehalosi* type T were adjusted to contain 1×10^8 CFU at the final vaccine formula, according to Abd El-Moneim et al., (2022).

2.5. E. Coli Antigenic Culture preparation (El Sayed, 2021):

E. coli K99 was cultivated for 24 h at 37°C on brain heart agar for incubation. The harvesting was done using normal saline, followed by inactivation with 0.5% formalin. The antigenic bacterial phase was adjusted to 1×10^9 CFU in the final vaccine formula.

2.6. Preparation of the vaccines:

In this research, three types of inactivated vaccines were prepared: Pasteurellosis vaccine (vaccine 1), the *E. coli* vaccine (vaccine 2), and the combined inactivated Pasteurellosis and *E. coli* vaccine (vaccine 3). All of them were adjuvanted with Montanide oil™ ISA 206 VG.
The preparation process was done according to the Montanide manufacturer (SEPPIC Co., France). The culture was mixed with Montanide™ ISA 206 VG at a 50% ratio using a low shear rate and a controlled temperature of 31°C (+/- 1°C).

2.7. Quality control tests of the prepared vaccines formula (Ashraf et al, 2018):
2.7.1. Sterility:

The prepared vaccines were examined for confirmation that they were free from contamination by bacteria and fungi. Inoculation of the prepared vaccines was done on nutrient agar, Sabouraud dextrose agar, and thioglycolate broth, followed by incubation for 72 hours at 37 °C. Also, cultivation was made for detection of *Mycoplasma* contamination on *Mycoplasma* agar, then incubated for 14 days at 37 °C at 5% CO2. The pure vaccines showed no growth on these media.

2.7.2. Safety test:

Fifteen Swiss white mice (5 for each vaccine) were injected with 0.2 ml of the prepared vaccines.

2.8. Experimental design:

Sheep at the age of 6 weeks, 20 males, healthy sheep were divided randomly into 4 groups (5 sheep per group). The first group was immunized with the *P. multocida* and *M. haemolytica* vaccine (vaccine 1). The second group was immunized with the *E. coli* vaccine (vaccine 2). The third group was immunized with *P. multocida, M. haemolytica,* and *E. coli* combined vaccine (vaccine 3). The fourth group was kept as a control group. The vaccinated sheep were injected subcutaneously with two doses (2 weeks apart between each dose) of the vaccines (1 ml/dose). Blood samples were collected before the 1st dose and 2nd doses, then monthly until the antibody titers began to decrease. The collected serum samples were used to measure the immune response of vaccinated groups using the indirect hemagglutination (IHA) test for *Pasteurella* and *Mannheimia* and the enzyme-linked immunosorbent assay (ELISA) test for *E. coli.*

2.9. The humoral immune response for evaluation of the prepared vaccines:
2.9.1. Evaluation of the humoral immune response of the vaccinated sheep against the Pasteurella and Mannheimia vaccines using indirect hemagglutination (IHA) test:

The test was done according to the OIE Terrestrial Manual 2008. Briefly, 2-fold serial dilutions of the collected sample sera, starting with 1/2, were prepared in stabilizer buffer pH 7.2 to give a final volume of 50 µl/well (microtiter plate), and 50 µl of sensitized RBCs were added to each well. Controls consisted of unsensitized erythrocytes plus test serum and sensitized erythrocytes plus diluent. The plates were shaken, then left at room temperature for approximately 2 hours, where the first reading was taken. The plates were then placed in the refrigerator until the next morning, when the second reading was taken.

2.9.2. Evaluation of the humoral immune response of the vaccinated sheep against E. coli using ELISA test:

The test was performed on the same serum sample, according to the method described by Voller et al. (1976)

and Briggs and Skeels (1984). The results were calculated according to the following formula: S/p (sample/positive) = sample mean - negative control/positive control- negative control.
Log10 titre = 1.09 (log10 S/P) + 3.63 titre = antilog10

2.10. Statistical analysis:

Results were declared as mean ± standard deviation (SD). One-way ANOVA test was used to calculate the difference between groups (P values) using SPSS program version 26 (IBM Corp., 2019).

3. RESULTS

3.1. Quality control testing of the prepared vaccines:
3.1.1. Sterility tests showed that all the prepared vaccines were free from bacterial, fungal, and mycoplasma contamination.
3.1.2. The prepared vaccines were proved to be safe; there were no local or systemic post-injection reactions for 15 days of clinical observation.

3.2. Indirect Hemagglutination (IHA) Test results for *P. multocida* and *M. haemolytica* vaccines (vaccines 1 and 3):

According to Table (1), there was no significant difference between the two vaccinated groups (vaccines 1 and 3) in all months. But a significant difference between the vaccinated and control groups was noticed. In all vaccinated groups, the titers of antibodies began to increase from the 1st week after vaccination and boostering at 2 weeks post-vaccination, reaching the highest level for *P. multocida* types A and B6 at the 4th month and type D at the 5th month (for vaccines 1 and 3, respectively). *M. haemolytica* type A reached the highest titer in the 3rd month, but *P. trehalosi* type T gave the highest titer in the 4th month.

3.3. ELISA test results for *E. coli* vaccines (vaccines 2 and 3):
According to table (2), there was no significant difference between the two vaccinated groups (vaccines 2 and 3) in all months. But a significant difference between the vaccinated and control groups was noticed. In all vaccinated groups, the titers of antibodies began to increase from the 1st week after vaccination and boostering at 2 weeks post-vaccination, reaching the highest level for the 3rd month *E. coli* vaccine (14507.2±57.6) and for the combined vaccine (14489.5±58.1).

Table 1 showing the comparison results between antibodies titers against *P. multocida* type A, D, and B6, *M. haemolytica* type A and *P. trehalosi* in the vaccinated groups (Pasteurella vaccine group 1, combined Pasteurella and E. coli vaccine group 3) and control group using the indirect hemagglutination test.

	Pasteurella Vaccine					Combined vaccine					Control
	P. multocida			*M. haemolytica*	*P. trehalosi*	*P. multocida*			*M. haemolytica*	*P. trehalosi*	
	A	D	B6	A	T	A	D	B6	A	T	
Day 0 vaccination	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]	2±0 [a]
2nd week post vaccination (boostering)	106. 7 ± 36.9[b]	74.7± 48.9 [b]	106.7± 36.9 [b]	106.7± 36.9 [b]	53.3± 18.5 [b]	53.3± 18.5 b	37.3 ± 24.4 [b]	53.3 ± 18.5 [b]	53.3± 18.5 [b]	26.7± 9.2 [b]	2±0 [a]
1st month	597.3± 391 [b]	149.3± 97.8 [b]	298.7± 195.5 [b]	426.7± 147.8 [b]	106.7± 36.9 [b]	298.7± 195.5 [b]	74.7 ± 48.9 [b]	149.3 ± 97.8 [b]	213.3± 73.9 [b]	53.3± 18.5 [b]	2±0 [a]
2nd month	853.3± 295.6 [b]	298.7± 195.5 [b]	426.7± 147.8 [b]	853.3± 295.6 [b]	256±0 [b]	426.7± 147.8 [b]	149.3 ± 97.8 [b]	213.3± 73.9 [b]	426.7± 147.8 [b]	128±0 [b]	2±0 [a]
3rd month	1718.6± 601.9 [b]	298.7± 195.5 [b]	853.3± 295.6 [b]	2048±0 [b]	426.7± 147.8 [b]	853.3± 295.6 [b]	149.3± 97.8 [b]	426.7± 147.8 [b]	1024±0 [b]	213.3± 73.9 [b]	2±0 [a]
4th month	2048±0 [b]	597.3± 391 [b]	1706.7± 591.2 [b]	2048±0 [b]	768± 443.4 [b]	1706.7± 591.2 [b]	298.7 ± 195.5 [b]	853.3 ±295.6 [b]	1706.7 ±591.2 [b]	384 ±221.7 [b]	2±0 [a]
5th month	1706.6± 591.2 [b]	1194.7± 782.1 [b]	1706.7± 591.2 [b]	1706.7± 591.2 [b]	256±0 [b]	853.3± 295.6 [b]	597.3 ±391 [b]	853.3 ±295.6 [b]	853.3 ±295.6 [b]	128±0 [b]	2±0 [a]
6th month	853.3± 295.6 [b]	298.7± 195.5 [b]	853.3± 295.6 [b]	853.3± 295.6 [b]	128±0 [b]	426.7± 147.8 [b]	149.3 ±97.8 [b]	426.7 ±147.8 [b]	426.7± 147.8 [b]	64±0 [b]	2±0 [a]
7th month	256±0 [b]	149.3± 97.8 [b]	256±0 [b]	213.3± 73.9 [b]	106.7± 36.9 [b]	170.7± 73.9 [b]	74.7 ± 48.9 [b]	149.3± 97.8 [b]	106.7± 36.9 [b]	53.3± 18.5 [b]	2±0 [a]
8th month	128±0 [b]	42.7± 18.5 [b]	128±0 [b]	106.7± 38.9 [b]	64±0 [b]	85.3± 36.9 [b]	37.3 ± 24.4 [b]	64 ±0 [b]	53.3± 18.5 [b]	42.7± 18.5 [b]	2±0 [a]

Data are presented as mean ±SD. Means with different superscript small letters indicate significant differences in the same raw between groups at $P < 0.05$ using a one-way ANOVA test.

Table 2 Showing the comparison results between antibodies titers against E. coli in the vaccinated groups (E. coli vaccine group 2, combined Pasteurella and E. coli vaccine group 3) and control group using ELISA test.

	E. coli vaccine	Combined Vaccine	Control
Day 0 vaccination	440.7±0.6 [a]	440.3±1.1 [a]	439.3±1.5 [a]
2nd week post vaccination (boostering)	2408.1±2.6 [b]	2390.4±0.5 [b]	441.0±1.0 [a]
1st month	4575.9±4 [b]	4558.2±1.0 [b]	440.1±1.0 [a]
2nd month	8258.4±3.2 [b]	8240.7±0.5 [b]	439.3±1.1 [a]
3rd month	14507.2±57.6 [b]	14489.5±58.1 [b]	440.3±1.1 [a]
4th month	9758.8±2.0 [b]	9741.1±1.0 [b]	438.3±1.1 [a]
5th month	9186.9±2 [b]	9169.3±1.1 [b]	441.0±1.0 [a]
6th month	8102.9±2 [b]	8085.3±1.1 [b]	439.3±1.1 [a]
7th month	4256.9±4 [b]	4239.2±1.0 [b]	440.3±1.1 [a]
8th month	3044.5±1.9 [b]	3026.9±3.5 [b]	439.7±0.6 [a]

Data are presented as mean ±SD. Means with different superscript small letters indicate significant differences in the same raw between groups at $P < 0.05$ using one-way ANOVA test.

4. DISCUSSION

In this study, *Pasteurella, Mannheimia*, and *E. coli* monovalent and combined vaccines were prepared and adjuvanted with Montanide™ ISA 206 VG. Sheep were grouped and vaccinated with two doses of each type of vaccine according to their group. The successfully combined vaccines decrease the stress on animals and workers and save time and expense.

In this study, the IHA test was used to evaluate the antibody titers acquired from vaccination with the *Pasteurella* and *Mannheimia* vaccines and the combined one, as shown in Table (1), revealed that the antibody titers increased gradually from the 1st week after vaccination in both vaccinated groups (1 and 3 groups) and boostering till reaching the highest titers, then began to decrease from the 5th and 6th months.

Statistically, there was no significant difference between the two vaccinated groups in the same month for all months. While there was a significant difference between the vaccinated and control groups. In this respect, Muenthaisong et al. (2021) prepared and evaluated the *P. multocida* and foot and mouth disease combined vaccine and concluded that there was a significant difference between the combined vaccine and the *P. multocida* vaccine. Also, the combined vaccine can be administered safely and produce a degree of immunogenicity with no adverse effects. Mori et al. (2019) prepared an inactivated vaccine against *H. somni*, *P. multocida*, and *M. haemolytica* and found that vaccination and boostering by *P. multocida* were significantly increased. Rahman et al (2016), who prepared the *P. multocida* vaccine from field isolates, cited that the passive hemagglutination (PHA) titers (134.86 ± 114.582 at 28 days' post-vaccination) from vaccinated cattle may be protectable against hemorrhagic septicemia disease. at the same respect Sarwar et al. (2015) prepared hemorrhagic septicemia (*P.multocida*) vaccines and evaluated them by IHA test, and they concluded that montanide adjuvant played a role in improving the quality of the vaccine and the vaccinated animals with a booster dose of the oil-adjuvant vaccine exhibited long-term immunity. At the same respect, Hanna et al. (2014) compared the bivalent clostridial and Pasteurella combined vaccine, *P. multocida* (types A, D, and *M. haemolytica*), and Clostridial vaccine in sheep and found no significant difference in antibody titers between the vaccines; and the combined vaccine was reliable for protection.

ELISA was used for evaluating the antibody titers produced from vaccination with *E. coli*. As shown in table (2), the titers of antibodies increased from the 1st week after vaccination and boostering in both vaccinated groups (2 and 3) until reaching the highest titers (3rd month), then began to decrease. Statistically, there was no significant difference between the two vaccinated groups in the same month for all months. In addition, there was a significant difference between the vaccinated and control groups. El-Sayed et al. (2011) prepared a combined inactivated bovine Rotavirus (BRV), Coronavirus, and *E. coli* strain K99 and cited that the antibody titers against *E. coli* revealed that the antibodies increased at the 4th-week post-vaccination and reached the highest titer at the 4th month, then began to decrease. Daoud et al. (2005), who prepared and evaluated a combined inactivated vaccine containing *E. coli* (K99), *C. perfringens*, Corona, and Rotaviruses, concluded that the maximal antibody titer obtained for *E. coli* was at the 8th-week post-vaccination. There was no immunological interference between *E. coli* bacterin and the other antigens in the inactivated vaccine.

5. CONCLUSIONS

The combined *Pasteurella, Mannheimia,* and *E. coli* vaccine is effective and more accessible to manipulate than a separate vaccine. Also, the combined vaccine had no adverse effect on the antibody titers compared to the results obtained from the separate vaccines.

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