2022, Scienceline Publication
World's Veterinary Journal
World Vet J, 12(3): 304-310, September 25, 2022
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2022.wvj38

Use of Inactivated *Corynebacterium pseudotuberculosis* as an Immunostimulant with Pneumobac Vaccine

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ORIGINAL ARTICLE
pii: S232245682200038-12
Received: 09 July 2022
Accepted: 23 August 2022

ABSTRACT

Sheep breeders in Egypt suffer from pneumonic pasteurellosis caused by *Pasteurella trehalosi*, *Pasteurella multocida*, and *Mannheimia haemolytica*. The disease is responsible for significant economic losses in the sheep industry according to the high mortality rate and reduced carcass values. Pneumobac® is the primary vaccine in Egypt used to control pasteurellosis in sheep. Therefore, the aim of the present study was to estimate the nonspecific immune stimulating impact of *Corynebacterium pseudotuberculosis ovis* against *Pasteurella* in sheep vaccinated with Pneumobac®. Nine sheep were classified into three groups, each with three animals. The sheep in the first and second groups were inoculated with the inactivated culture of Pneumobac® and a combined inactivated culture of Pneumobac® with *Corynebacterium pseudotuberculosis ovis* bacterin, respectively. The third group was nonvaccinated and kept in control. Indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA) were used to measure the humoral immune response to the produced vaccines. The results of the present study confirmed that the antibodies titer against *Pasteurella multocida* type A, D, and B6, *Pasteurella trehalosi* type T, and *Mannheimia haemolytica* type A significantly increased in sheep vaccinated with a combined vaccine (Pneumobac® and *Corynebacterium pseudotuberculosis ovis* bacterin), compared to those vaccinated with Pneumobac® alone. It was concluded that the addition of *Corynebacterium pseudotuberculosis ovis* bacterin to inactivated Pneumobac® vaccine could increase the immune response against pneumonic pasteurellosis.

Keywords: *Corynebacterium pseudotuberculosis*, *Pasteurella multocida*, Pasteurellosis, Pneumobac®

INTRODUCTION

Pneumonic pasteurellosis is an infectious disease caused by *Pasteurella* species that is responsible for the mortality of 25-30% and morbidity up to 50% in affected adult animals or lambs (De Alwis, 1999; James et al., 2015), especially those who have not received a sufficient colostrum amount (Kebkiba, 2021). The disease is characterized clinically by anorexia, pyrexia, occulonasal discharges, rapid shallow respiration, and pathologically by pleuritis and pneumonia (Sahay et al., 2020). Ovine pneumonia is responsible for worldwide economic loss in the sheep industry (Singh et al., 2019; Sahay et al., 2020). The microbes are commensal in the lung without causing any pathology. However, the disease appears under stress conditions, such as transportation, weaning, and diet changes (Akane et al., 2022). The disease develops in case of respiratory tract viral infection that becomes complicated by infection with *Pasteurella multocida* (*P. multocida*), *Mannheimia haemolytica* (*M. haemolytica*), and *Pasteurella trehalosi* (*P. trehalosi*) or other bacterial species, such as *Bordetella parapertussis* and *Mycoplasma ovipneumoniae* (Naglaa et al., 2019; Alarawi and Saeed, 2021). *Pasteurella and Mannheimia* bacteria are bipolar coccobacillus, gram negative, nonmotile, and facultative anaerobic bacteria (Sahay et al., 2020). Pneumonic pasteurellosis is a highly contagious disease that affects various animal species, including rodents, cattle, goats, sheep, turkeys, and rabbits. Conventional preventive measures are expensive, complex, and ineffective (Mostaan et al., 2020). In most cases of pasteurellosis, chemotherapeutic treatment is only effective for a short time before the disease reappears, as well as some drugs are toxic to human consumers; therefore the use of vaccination strategies is the best method used for control of disease in the developing nation (Ahmad et al., 2018). Recently, researchers have been directed toward producing more potent and effective vaccines (Mostaan et al., 2020). The vaccine's efficacy is determined by a variety of factors, including the amount and type of antigen used, as well as the presence of adjuvants to enhance the immunogenicity of the developed vaccine (Mandado, 2019). *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) *ovis* has shown its ability as a nonspecific immune stimulant capable of elevating sheep's resistance to artificial infection with potential pathogens, in a method similar to that produced by Bacille Calmette Guerin (BCG). The waxy elements in the *C. pseudotuberculosis* structure stimulate antibody production in the same way as *Mycobacterium* waxes associated with Freund's complete adjuvant do. Freund's

To cite this paper: Abd El-Moneim WSA, Mohamed MM, Khedr MMS, EL-Rawy EM, and Mwafy A (2022). Use of Inactivated *Corynebacterium pseudotuberculosis* as an Immunostimulant with Pneumobac Vaccine. *World Vet. J.*, 12 (3): 304-310. DOI: https://dx.doi.org/10.54203/scil.2022.wvj38

complete adjuvant can improve the antibody response to soluble protein antigens by stimulating the innate immune system (Barakat et al., 1984; Trott et al., 2008). Pneumobac® is an inactivated oil adjuvant pasteurellosis vaccine that is the most effective used to control sheep pneumonic pasteurellosis (El-Kattan et al., 2019).

The present study aimed to evaluate *C. pseudotuberculosis* as a nonspecific immune-stimulating impact against *Pasteurella* in sheep that received the Pneumobac® vaccine.

MATERIALS AND METHODS

Ethical approval

All methods were carried out in compliance with the National Research Committee of Egypt's ethical guidelines and authorized by the Animal Care Committee (Central Laboratory for the Evaluation of Veterinary Biologics, Cairo, Egypt, Code No. 326).

Experimental animals

Nine healthy Egyptian native breeds of sheep (Rhamani) were selected after a clinical examination by an expert veterinary team from the Faculty of Veterinary Medicine and the Veterinary Serum and Vaccine Research Institute, Giza, Egypt, to confirm that all selected sheep were healthy and free from other abnormalities. The sheep were bought from Nubariah local farm for domestic animals in Giza Governorate and with an average body weight of about 25 kg and two months of age. The sheep were divided into three groups, each group containing three animals. They were housed in the farm's experimental housing (16 feet wide × 12 feet long; temperature of 61-81°F, and humidity of 30-70%) and were quarantined for latent diseases before the study. The sheep were fed on high roughage, stored hay, and low moisture grass silage. The water and food were continuously available, and the experiments were performed after two weeks of adaptation.

Bacterial strains

Pasteurella multocida type B6 standard strain, *P. multocida* types A and D, *M. haemolytica* type A, and *P. trehalosi* type T were locally isolated and identified by Prof. Eman (El-Sawah and Eman, 2010) and were used for vaccine preparation. *Corynebacterium pseudotuberculosis ovis* strain was locally isolated from Egyptian native sheep and was fully identified according to Koneman et al. (1997) and MacFaddin (2000). The *C. pseudotuberculosis ovis* strain was formalin-inactivated for the preparation of bacteria.

Synthesis of *Corynebacterium pseudotuberculosis* bacterin

Corynebacterium pseudotuberculosis ovis bacterin was performed according to the method of Auad et al. (2018). The *C. pseudotuberculosis* isolate was grown in brain heart infusion broth and cultured at 37°C in a shaking incubator for 48 hours. The bacterial cells were collected by centrifugation in a cooling centrifuge at 4000 rpm for 10 minutes. The obtained sediments were washed twice with sterile distilled water, and then the pellet was washed once with 100% acetone before being washed twice with ether and dried by air. The bacterial cells were suspended in formalin (1%) and kept overnight for complete inactivation. The inactivated bacteria were grown on brain heart infusion agar to ensure sterility.

Preparation of Pneumobac® vaccine

Formalin inactivated culture of *P. multocida* capsular biotypes A, D, and B6 contain 1×10^7 C.F.U. for each were mixed with equal volumes of formalized local isolates of *M. haemolytica* type A and *P. trehalosi* type T contains 1×10^8 C.F.U. for each. An equal amount of the above-mentioned culture was mixed using magnetic stirring (MMS-3000, Biosan) at 300 rpm according to the OIE Manual (2021) method.

Preparation of combined Pneumobac® and *Corynebacterium pseudotuberculosis ovis* bacterin vaccine

An equal amount of the inactivated Pneumobac® (VSVRI, Egypt) culture was mixed thoroughly with an equal amount of 1.5×10^8 C.F.U. of *C. pseudotuberculosis ovis* bacterin (50/50), according to the method of OIE Manual (2021).

Quality control of the produced vaccines

The produced vaccines were submitted to sterility and safety testing in accordance with the OIE Manual (2021).

Experimental design

At the age of two months, nine healthy sheep were divided into three groups (three sheep/each group). The first group was immunized with an inactivated culture of Pneumobac®, and the second group was immunized with the

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combined inactivated culture of Pneumobac® and *C. pseudotuberculosis ovis* bacterin. The third group was considered to control nonvaccinated. The vaccinated sheep were injected subcutaneously (18 G and1/4 Inch Needle length) with two doses of vaccine (1ml/dose), the one-month interval between the two doses, and blood was collected monthly until they reached 9 months of age. The collected serum samples were used to measure the immune response of vaccinated groups using enzyme linked immunosorbent assay (ELISA) and Indirect haemagglutination test (IHA).

Indirect haemagglutination test

The IHA test for detection of the *Pasteurella* antibodies was done, followed by the administration of *Pasteurella* vaccines, according to Ferede et al. (2013).

Enzyme-linked immunosorbent assay

The ELISA test was performed for detection of the Pasteurella antibodies was done followed by the administration of Pasteurella vaccines according to Takada-Iwao et al. (2007).

Statistical analysis

The statistical analysis of the resulted data was done on Minitab 14® for statistical analysis. Statistical analysis included IHA tests (tables 1 and 2), ELISA tests (tables 3 and 4), and the comparative analysis was performed by statistical T-test, Pearson correlation coefficient. Moreover, all results were assessed by one-way ANOVA with a p-value < 0.05 considered significant (Cornell, 1981).

RESULTS

Quality control of the produced vaccines

The prepared *Pasteurella* vaccines were confirmed to be safe without any morbidity or mortality when inoculated in white Swiss mice and sterile and free from any bacterial and fungal contaminants.

Indirect haemagglutination test

The humoral immune response of sheep immunized with different *Pasteurella* vaccines (Pneumobac® and combined Pneumobac® and *C. pseudotuberculosis ovis* bacterin vaccines) by using IHA. As shown in Table 1, the mean antibodies titer against *P. multocida* type A, D, and B6 in the group of sheep vaccinated with the combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) was 160, 154, and 196, respectively. The mean of antibodies titer against *P. multocida* type A, D, and B6 in the sheep group vaccinated with Pneumobac® alone were 82, 88, and 101, respectively. Moreover, the results in Table 2 demonstrated that the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T by using the IHA were 208 and 224, respectively, in the group of sheep vaccinated with a combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin). Moreover, the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with Pneumobac® were 116 and 114, respectively.

Table 1. Level of antibodies titer against *Pasteurella multocida* type A, D, and B6 of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by the indirect haemagglutination test

Vaccinated groups / Interval time of serum collection		Pneumobac®			Combined Pneumobac® and *Corynebacterium*			Control		
		A	D	B6	A	D	B6	A	D	B6
Pre-vaccination		2	2	2	2	2	4	0	0	0
First dose of vaccine*	First month after first vaccine	32	16	16	32	32	32	2	2	0
Booster dose of vaccine*	Second month	32	64	32	64	64	128	4	0	0
	Third month	128	128	128	256	256	512	2	0	2
	Fourth month	128	256	256	256	512	512	4	2	2
	Fifth month	256	128	256	512	256	256	2	2	0
	Sixth month	128	128	128	256	128	256	2	4	0
	Seventh month	64	64	128	128	128	128	0	0	2
	Eighth month	32	64	32	64	128	64	2	0	4
	Ninth month	16	32	32	32	32	64	0	2	0
	Overall mean	82	88	101	160	154	196	1.8	1.2	1

Number of colony count = 1×10^7 C.F.U. /ml

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Table 2. Level of antibodies titer against *Mannheimia haemolytica* type A and *Pasteurella trehalosi* type T of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by the indirect haemagglutination test

Vaccinated groups / Interval time of serum collection		Pneumobac®		Combined Pneumobac® and *Corynebacterium*		Control	
		A	T	A	T	A	T
Pre-vaccination		2	2	2	2	0	0
First dose of vaccine*	First month after first vaccine	32	16	32	64	0	2
Booster dose of vaccine*	Second month	64	64	128	128	4	2
	Third month	256	256	256	256	2	2
	Fourth month	256	256	256	512	2	2
	Fifth month	256	256	512	512	0	0
	Sixth month	128	128	256	256	0	2
	Seventh month	64	64	256	256	0	0
	Eighth month	64	64	256	128	2	2
	Ninth month	32	32	128	128	2	2
	Overall mean	116	114	208	224	1.2	1.4

Number of colony count = 1×10^8 C.F.U. /ml

Enzyme linked immunosorbent assay

As shown in Table 3, the overall mean of antibodies titer against *P. multocida* type A, D, and B6 in sheep vaccinated with combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) by using ELISA were 433, 510, and 514, respectively. The mean of antibodies titer against *P. multocida* type A, D and B6 in the group of sheep vaccinated with Pneumobac® were 314, 337, and 333, respectively. The results in Table 4 demonstrated that the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with combined vaccine was 541 and 612, respectively. While, in the sheep group that was vaccinated with Pneumobac® vaccine, the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T were 398 and 407, respectively.

Table 3. Level of antibodies titer against *Pasteurella multocida* type A, D and B6 of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by enzyme linked immunosorbent assay

Vaccinated groups / Interval time of serum collection		Pneumobac®			Combined Pneumobac® and *Corynebacterium*			Control		
		A	D	B6	A	D	B6	A	D	B6
Pre-vaccination		10	20	10	20	10	20	0	0	0
First dose of vaccine*										
First month after first vaccine		100	250	100	180	300	290	0	0	0
Booster dose of vaccine*	Second month	205	306	202	295	466	583	0	0	0
	Third month	460	430	563	590	798	770	20	20	20
	Fourth month	505	450	710	700	765	798	0	0	0
	Fifth month	550	554	512	778	800	798	20	20	10
	Sixth month	490	500	415	655	690	798	0	0	20
	Seventh month	470	415	340	555	498	360	20	20	20
	Eighth month	200	270	210	355	415	360	20	0	0
	Ninth month	150	170	270	202	355	360	0	20	0
	Overall mean	314	337	333	433	510	514	8	8	7

Number of colony count = 1×10^7 C.F.U. /ml

Table 4. Level of antibodies titer against *Mannheimia haemolytica* type A and *Pasteurella trehalosi* type T of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by enzyme linked immunosorbent assay

Vaccinated groups / Interval time of serum collection		Pneumobac®		Combined Pneumobac® and *Corynebacterium*		Control	
		A	T	A	T	A	T
Pre-vaccination		20	20	20	20	0	0
First dose of vaccine*							
First month after first vaccine		220	102	280	230	20	0
Booster dose of vaccine*	Second month	370	454	495	680	0	2
	Third month	453	576	590	810	20	2
	Fourth month	660	608	760	998	20	2
	Fifth month	745	650	787	790	20	0
	Sixth month	523	506	690	699	0	2
	Seventh month	440	440	655	670	0	0
	Eighth month	280	400	576	620	20	2
	Ninth month	270	318	555	600	0	2
	Overall mean	398	407	541	612	10	1.4

Number of colony count = 1×10^8 C.F.U. /ml

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DISCUSSION

For Egyptian sheep producers, pulmonary pasteurellosis is a complex disease with a morbidity of 50% and a death rate 25-30% if the animals are not treated in the early stage of infection, resulting in significant financial losses (De Alwis, 1999; El-Sawah and Eman, 2010; James et al., 2015). *Mannheimia haemolytica* and *P. multocida* are the most common organisms that cause pneumonic pasteurellosis in sheep (Taye et al., 2019). These organisms are usually found in the upper respiratory tracts of healthy animals as normal inhabitants; however, when the animal's immune system is compromised by stressors including travel, crowding, a lack of water, and concurrent viral, *Mycoplasma*, and lungworms infections, they can cause significant illness (Asfaw et al., 2022; Getnet et al., 2022). Because prevention is the most likely way to control the disease, vaccines will be of great value in protecting animals from pasteurellosis (Ismail et al., 2018). The work aimed to study the nonspecific immune-stimulating effects of *C. pseudotuberculosis ovis* bacterin against *Pasteurella* species in sheep vaccinated with Pneumobac®.

The immune response of the sheep immunized with combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) and Pneumobac® was evaluated by using an IHA as shown in Table 1, 2. Using the IHA test, the overall mean of circulating antibodies titers against *P. multocida* types A, D, and B6 in sheep vaccinated with a combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) showed a significant increase compared to sheep vaccinated with Pneumobac® alone ($p < 0.05$). In addition to, the overall mean of circulating antibodies against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with a combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) was significantly increased than the group of sheep vaccinated with Pneumobac® alone ($p < 0.05$). While, the result in Table 3 demonstrated the mean level of antibodies titer against *P. multocida* type A, D, and B6 in the group of sheep vaccinated with the combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) using ELISA was significantly higher than the group of sheep vaccinated with Pneumobac® alone ($p < 0.05$). The results in Table 4 indicated that the overall mean of antibody titer against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with a combined vaccine was significantly increased than the group of sheep vaccinated with Pneumobac® ($p < 0.05$). The findings are in accordance with those of Barakat et al. (1984) who showed that *C. pseudotuberculosis ovis* has an immune-stimulating impact when used as an adjuvant with various antigens, including egg albumin, Food and mouth viral disease, and *Salmonella typhimurium*. In addition, the above results are inconsistent with those obtained by Eggleton et al. (1991) found that the protective efficacy of the vaccines was not improved by *C. pseudotuberculosis ovis* bacterin, but considered the toxin produced by *C. pseudotuberculosis* to be the main factor responsible for protection. In Egypt, Marwah et al. (2015) showed that the phospholipase D exotoxin of *C. pseudotuberculosis* was an effective nonspecific immune stimulant that could be used in combination with inactivated Newcastle and Mycoplasma vaccines to elicit an early, better, and longer immune response.

Freund (1956) and Barakat et al. (1984) explain the nonspecific ability of *C. pseudotuberculosis ovis* to raise the resistance of sheep to artificial infection with potential pathogens in a manner comparable with that produced by BCG, as the waxy material in the cell wall structure of *C. pseudotuberculosis ovis* appears to stimulate antibody production in the same way that Mycobacteria waxes do.

CONCLUSION

It is concluded that the *C. pseudotuberculosis ovis* bacterin has nonspecific immune-stimulating effects against *Pasteurella* species in sheep vaccinated with inactivated Pneumobac® and produces a better immune response. Moreover, further studies on the nonspecific immune stimulating effect of inactivated *C. pseudotuberculosis ovis* on other bacterial and viral species are recommended to improve vaccine potency. Also, more immunological studies on the *C. pseudotuberculosis* bacterin and its toxin are required to determine how they work to enhance immunity against other bacteria. Finally, the obtained results are essential for developing the *Pasteurella* vaccine.

DECLARATIONS

Acknowledgments

The author's great thankful to the Veterinary Serum and Vaccine Research Institute (VSVRI), Agricultural Research Center (ARC), Egypt, for their financial support and materials provided during the practical part of this research.

Authors' contribution

In the present study, all authors contributed to this research work. Eman Mohamed EL-Rawy did an experimental design. Wafaa Sayed Ahmed and Marwah Mohamed Mohamed prepared the vaccines. Marwa Magdy Sayed Khedr and Abeer Abdelsadek Ahmed Mwafy collected the blood samples and did the statistical analysis. Wafaa Sayed Ahmed,

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Abeer Abdelsadek Ahmed Mwafy, Marwah Mohamed Mohamed, and Marwa Magdy Sayed Khedr did the evaluation of the immune response. All authors contributed equally to the writing and review of the manuscript, as well as to the collection of papers related to the research subject. The final version of the manuscript to be published in the present journal was read and approved by all authors.

Competing interests

The authors declare that there is no conflict of interest.

Ethical considerations

Ethical concerns, such as redundancy, misconduct, publishing consent, the fabrication or falsification of data, multiple submission or publication, and plagiarism have been verified by the authors.

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To cite this paper: Abd El-Moneim WSA, Mohamed MM, Khedr MMS, EL-Rawy EM, and Mwafy A (2022). Use of Inactivated *Corynebacterium pseudotuberculosis* as an Immunostimulant with Pneumobac Vaccine. *World Vet. J.*, 12 (3): 304-310. DOI: https://dx.doi.org/10.54203/scil.2022.wvj38

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