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Original Research Article

Field evaluation of inactivated *Corynebacterium ovis* vaccine in sheep prepared from a local isolate

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ABSTRACT

Corynebacterium pseudotuberculosis vaccine was prepared from a local field isolate. Vaccination of sheep with 50µg PLD toxoid and 10 mg bacterin adjuvanted with Montanide oil improved the levels of immune responses of sheep. In many countries, inactivated *C. pseudotuberculosis* adjuvant vaccines have been used for prevention and control of caseous lymphadenitis in sheep. However, the efficacy was variable. The aim of the present study was directed to prepare and evaluate the potency of an inactivated *C. pseudotuberculosis* vaccine using Montanide ISA206. Sheep were vaccinated with 1st dose of 2ml containing 10 mg bacterin and 50µg toxoid and Montanide ISA 206 oil adjuvant and boostered with the same dose 15 days Apart. Evaluation of post vaccinal cellular immune response with lymphocyte proliferation assay and humoral immune response using ELISA was carried out. Cell mediated immune response of vaccinated sheep reached its peak 0.445 by 1st week post the second vaccination. The level of humoral immune response showed optical density of 1.005 by 1st week post the second vaccination. Challenge test was done in all sheep four weeks after the second dose of vaccination. Three sheep from vaccinated and three sheep from non-vaccinated groups were slaughtered and necropsied 150 days post challenge. The results revealed 75% protection percentage against challenge while unvaccinated challenged sheep showed 9% protection. Statistical analysis indicated that the vaccine assessed a significant level of cellular and humoral immunity.

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1. Introduction

Caseous lymphadenitis is a serious disease affects small ruminants worldwide, caused by *Corynebacterium* (*C.*) *pseudotuberculosis*, which is a mycolic acid containing facultative intracellular actinomycete associated with the development of abscesses in infected small ruminants. After initial infections, lesions of such disease may not be observed up to several months, and the disease is manifested in two different forms: superficial or external caseous lymphadenitis including abscesses in superficial lymph nodes and subcutaneous tissue; and visceral or internal caseous lymphadenitis including abscesses in internal organs (Stoops et al., 1984; Paton et al., 1994; Williamson, 2001). The disease causes significant economic losses due to decreased meat production, damaged wool and leather and decreased reproductive efficiency.

Once the disease is established in a flock, eradication becomes difficult because antimicrobial therapy is not effective. The most reliable control strategy for such disease based on identifying and removing the infected animals (Stanford et al., 1998). So, massive vaccination may be necessary to reduce the disease prevalence.

The causative agent of the disease was firstly recovered in Egypt by Carpano (1934)**,** then demonstrated by several authors (Awad et al., 1977; Nashed and Mahmoud, 1987; Mubarak et al., 1999; Abou Zaid et al., 2001; Soheir, 2006; Manal, 2007). For many decades, researchers have attempted to develop an ideal vaccine for the prevention of caseous lymphadenitis in sheep. In many countries, killed *C. pseudotuberculosis* adjuvant vaccine has been used for prevention and control of the disease, but the efficacy was variable and results of protection rates mostly varied. Many scientists suggested that efficacy of killed adjuvant vaccines depend mainly on the used adjuvant which is safe for use and able to enhance immunogenicity of the vaccine (Cameron and Fuls, 1973; Brogden et al., 1996; Cameron et al., 1998).

The aim of the present study was directed to prepare an inactivated adjuvant vaccine from killed local isolated *C. pseudotuberculosis* field strain and evaluation of their potency in sheep. This objective could be fulfilled through isolation of *Corynebacterium pseudotuberculosis* field strain from sheep with complete identification of the obtained strain, preparation of phospholipase-D exotoxin from culture filtrate of the obtained *Corynebacterium pseudotuberculosis* field isolates, preparation of inactivated *Corynebacterium pseudotuberculosis* (bacterin) by using binary ethyleneimine, preparation of inactivated *Corynebacterium pseudotuberculosis* vaccine by using Montanide ISA206 and evaluation of the potency of the prepared vaccine in sheep.

2. Materials and methods

2.1. Animals

One hundred native sheep of about 6-8 months of age in Ismailia sheep farm were apparently healthy with no history of caseous lymphadenitis and all sheep used for the evaluation of the prepared vaccine showed negative ELISA. One hundred sheep were divided into two groups where the first Group, 90 sheep were vaccinated with the prepared and the other Group, 10 sheep were kept as control.

2.2. Bacteria

A local isolate of *C. pseudotuberculosis* was used to prepare an inactivated vaccine.

2.3. Vaccine preparation

2.3.1. Inactivation of *Corynebacterium pseudotuberculosis* using binary ethyleneimine

Ten ml of Binary ethyleneimine solution (30%) conc. were added to 1000 ml of bacterial suspension to get a final concentration of0.03% and was incubated at 37°C and checked as possible every one hour. The viability was tested every 24 hours on brain heart infusion agar plates. The excess of binary ethyleneimine was neutralized by sodium thiosulphate according to (Farid et al., 1979).

2.3.2. Preparation of culture filtrated toxoid

The culture filtrate containing PLD toxin was prepared using a minimum essential synthetic media (MEM) according to Moura-Costa et al. (2002) and Brain heart infusion media according to Brown et al. (1986).

The phospholipase D exotoxin was inactivated by addition of formalin 0.1% to the concentrated culture filtrate containing phospholipase D exotoxin (50 ug / ml) then mixed well and leaved for 24 hours at room temperature. The absence of toxin activity in toxoid preparation had been demonstrated as described by (Muckle and Gyles, 1983) in five mice. Merthiolate was added to the concentrated filtrate to a final concentration of 1:10.000 and the concentrated filtrate was stored in sterile prescription bottle under 4 °C refrigeration until needed.

2.3.3. Montanide ISA 206 oil adjuvant

It was prepared by emulsifying water phase containing phospholipase-D toxoid and Bacterin with Montanide ISA206 oil in ratio 1:1 (W/W) according to (Lyer et al., 2001). Dose in sheep for such vaccine is 2ml.

2.4. Evaluation of *Corynebacterium pseudotuberculosis* vaccine

It was performed according to Office International Des Epizooties (OIE) (2008) including purity; safety and potency tests.

2.5. Animal vaccination

Sheep were divided into two groups as follow: Group 1 contains 90 sheep which were vaccinated with 1st dose of 2 ml containing 10 mg bacterin + 50 µg pld toxoid + Montanide ISA 206 oil adjuvant and boostered with the same dose 15 days apart while, Group 2 contains 10 sheep which were kept as control.

2.6. Evaluation of protection efficiency of prepared *Corynebacterium pseudotuberculosis* vaccine

2.6.1. Determination of the cellular immune response through lymphocyte proliferation assay

It was applied according to Lee (1984).

2.6.2. Determination of humoral immune response using ELISA

It was carried out according to Menzies et al. (1994).

2.6.3. Challenge test

Evaluation of vaccine potency was carried out by challenge exposure test and necropsy examination of the tested animals according to Johnson et al. (1993). Challenge test was done in all sheep four weeks after the second dose of vaccination. Each sheep was inoculated with 1ml of 4×10^6 colony forming unit intradermally at the right paralumbar fossa (challenge exposure test).

2.6.4. Necropsy examination

It was done for slaughtered three challenged and three Control sheep was carried out 150 days after the challenge (Paule et al., 2003). All organs and all detectable lymph nodes were macroscopically evaluated for the presence of abscesses and score of infection was calculated (Piontkowski and Shivers, 1998).

3. Results

Table 1. The Amount of PLD toxin secreted in Minimum Essential Media (MEM) filtrates and brain heart culture filtrates concentrated by lypholization (freeze drying) and Millipore ultra-filtration system.

Detected items	Amount of PLD toxin			
	Batch-1 using lyophilization system		Batch-2 using Millipore ultrafiltration system	
	MEM	Brain Heart	MEM	Brain Heart
% in lane	22.16%	43.79%	21.173%	42.22%
Total protein of concentrated culture filtrate	27 ug/ml	70 ug/ml	29 ug/ml	74 ug/ml
Amount of PLD in concentrated filtrate	5.75 ug/ml	30.56 ug/ml	5.80 ug/ml	30.13 ug/ml

Table 2. Inactivation of *Corynebacterium pseudotuberculosis* using different concentrations of BEI.

Time in hours post inactivation	Inactivation rate of *Corynebacterium pseudotuberculosis* using different concentrations of BEI		
	0.01% BEI	0.02% BEI	0.03% BEI
0	+++	+++	+++
24	+++	++	+
48	++	+	-
72	+	-	-
96	+	-	-
120	-	-	-

The degree of growth on brain heart infusion agar plates was evaluated as follow:
+++ = Profuse ++ = Medium + = Low - = No growth = Complete inactivation

Table 3. Cell mediated immune response of sheep vaccinated with 1st dose of 2ml containing 10 mg bacterin and 50g toxoid adjuvanted by Montanide ISA 206 and boostered with the same dose 15 days by expressed by mean optical density values.

Groups	Cell mediated immune response post vaccination						
Time	0 day	1 week	2 weeks	The second dose of Vaccination	3 weeks	4 weeks	5 weeks
Vaccinated Group	0.165	0.340	0.445		0.493	0.390	0.303
Control group	0.155	0.160	0.161		0.139	0.160	0.147

Table 4. Humoral immune response of sheep vaccinated with 1st dose of 2ml containing 10 mg bacterin and 50g toxoid adjuvanted by Montanide ISA 206 and boostered with the same dose 15 days by expressed by mean optical density values of indirect ELISA.

Groups		Humoral immune response post vaccination						
Time		0 day	2 weeks	3 weeks	The second dose of Vaccination	4 weeks	6 weeks	8 weeks
Vaccinated	Group	0.475±0.067[a]	1.038±0.13[b]	1.40±0.05[b]		1.045±0.08[b]	0.74±0.06[b]	0.67±0.03[b]
Control	Group	0.403±0.01[a]	0.393±0.03[a]	0.431±0.02[a]		0.376±0.02[a]	0.39±0.001[a]	0.360±0.006[a]

The superscript alphabetical a and b indicate significant difference between vaccinated group and control group by using t-test.

Table 5. Scores of lesions detected at post mortem examination of challenged sheep group and unvaccinated control sheep group.

Sheep group	Animal Number	External lymph nodes				Internal lymph nodes	Total score of infected lymph nodes	Protection (%)
		RPS	LPS	RPF	LPF			
Vaccinated Group	1	0/3	0/3	2/3	0/3	3/3	11/45	75%
	2	0/3	0/3	2/3	0/3	3/3		
	3	0/3	1/3	0/3	0/3	0/3		
Control Group (non-vaccinated)	1	3/3	2/3	3/3	3/3	3/3	41/45	9%
	2	3/3	2/3	3/3	3/3	3/3		
	3	3/3	3/3	3/3	3/3	3/3		

RPS: Right prescapular lymph node, LPS: Left prescapular lymph node, RPF: Right prefemoral lymph node, and LPF: Left prefemoral lymph node

4. Discussion

In the present work, the yield of PLD toxin using brain heart infusion media was about 6 times more

than that using MEM synthetic media (Table 1) which agreed with those obtained by Paule et al. (2003) and Soheir (2006). It was found that the concentrated amount of PLD toxin did not affected by the use of concentration method, although Millipore ultra-filtration was a practically suitable for vaccine production in accordance with that obtained by Abd El-Fattah (1996). Complete inactivation of *C. pseudotuberculosis* using 0.01%, 0.02% and 0.03% BEI was achieved after 4 days, 2 days and 1 day respectively (Table 2). Following up the cell mediated immune response of vaccinated sheep it was found that the optical density of cell mediated immune response of vaccinated sheep with 50µg toxoid with 10 mg formalized bacterin adjuvanted with Mantonid oil vaccine increased in the second week of vaccination and reached its peak (0.445) by the 1st week post the second dose (3rd week post the first vaccination) (Table 3). ELISA results showed that the highest optical density level of humoral immune response showed optical density of 1.005 by the 1st week post the second vaccination (3rd week post the first vaccination) (Table 4).

On challenge of vaccinated and unvaccinated sheep, rectal temperature was recorded where all sheep experienced a transient febrile response (39.5-40.3°C) within 24 hours post challenge. Meanwhile the control group showed 41°C prolonged for 3 days, then decreased gradually to the normal level. Three sheep from challenged vaccinated group and three sheep from control group were slaughtered after 150 days pot challenge and the post mortem findings and its evaluation (Table 5). Sheep vaccinated with 50µg toxoid with 10 mg bacterin adjuvanted with Mantonid oil showed 75% protection against challenge while unvaccinated challenged sheep showed 9% protection (Table 5).

The protection percentages of vaccinated sheep group against experimental infection was 75% while the infection rate in unvaccinated sheep (control group) was 91% indicating that the prepared vaccine is safe and potent sufficient to protect sheep. Nearly similar results were reported by Hodgson et al. (1999) and Piontkowski and Shivers (1998) who reported 100% infection in unvaccinated sheep.

Vaccination of sheep with concentrated culture filtrate toxoid plus whole cell bacterin adjuvanted with Montanide ISA 206 elucidated a protection percentage of 75% (Sedky, 2006) used a vaccine composed of recombinant mutant PLD toxoid plus formalized cells treated by acetone and ethyl ether and adjuvanted with paraffin mineral oil which elicited 62% protection. Our study resulted in protection 75% which might be attributed to other culture filtrate toxins such as 40 kDa toxin, Aro Q toxin and cell wall lipids in addition to the type of used adjuvant. Such finding agreed with Hodgson et al. (1999) who had protection 44% with genetically inactivated phospholipase-D exotoxin and 95% protection by conventional formalin inactivated vaccine. Meanwhile, the same hypothesis of Eggleton et al. (1991b) and Fontaine et al. (2006) supposed that the toxoid vaccines prepared from *C. pseudotuberculosis* culture supernatant routinely contained other excreted secreted antigens, somatic and cell wall antigens which stimulate the protective immunity responses. The use of serial of oil adjuvants of Montanide series (Montanide ISA 25, 70, 57 and 206) resulted in enhancement of the immune response and these new oil formulations have favorable characteristics of low viscosity, lower reactivity and high potency (Abdel-Wanis et al., 2001; Abdel Hady, 2002). Meanwhile, Youssef (2004) recorded a lower protection (66%) when he used BCG dissolved in recombinant mutated phospholipase-D subcutaneously. This could be explained as he did not use oil adjuvant with the vaccine. In the current study, sheep were immunized with 50 ug phospholipase-D concentrated culture filtrate and 10 mg bacterin adjuvanted with Montanide ISA 206 producing 75% protection and there was no abscessation at vaccination injection site opposite to that given by Brogden et al. (1990) who revealed that vaccines containing 10 mg of the whole cell induced sterile abscesses detectable at the vaccination site in vaccinated lambs and when the concentration of whole cells lowered to one mg, the vaccine induced immunity in lambs without induction of sterile abscesses at the inoculation site.

5. Conclusion

It is concluded that the prepared inactivated *C. pseudotuberculosis* vaccine adjuvanted with montanide oil ISA 206 proved to be fully safe and highly potent and could be used for prevention and control of CLA in sheep.

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