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TRIALS OF PREPARATION AND EVALUATION OF CORYNEBACTERIUM PSEUDOTUBERCULOSIS VACCINES IN SHEEP

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A B S T R A C T

Different formulae of oil adjuvant vaccine were prepared from killed local *isolated Corynebacterium pseudotuberculosis field isolate* and their potency was evaluated in Guinea-pigs and sheep. The cellular and humeral immune response of vaccinated sheep and Guinea pigs were evaluated. The results showed that bacterin and PLD toxoid adjuvanted Montanide oil 206 *Corynebacterium pseudotuberculosis* is the most suitable vaccine. BCG could be helpful when used before vaccination of sheep with 50 µg PLD toxoid and 20 mg formalized bacterin adjuvanted Montanide oil to improve the levels of immune response of sheep. The results of postmortem examination of vaccinated and challenged Guinea pigs showed that the mean number of abscesses in skeletal muscles, lymph nodes and internal organs varied to lesser extent between groups vaccinated and control group. All sheep were slaughtered after 150 days and the post mortem examination showed that the different vaccinated sheep groups revealed protection percentages against experimental infection of 71.2-78% while the infection in non-vaccinated sheep was 91%. This results indicated that the prepared vaccine formulae are safe and potent to protect sheep.

KEY WORDS: *Corynebacterium pseudotuberculosis*, Guinea-pigs, Sheep, Vaccine

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1.INTRODUCTION

Caseous lymphadenitis is a serious disease, commonly occurs in small ruminants all over the world, caused by *Corynebacterium pseudotuberculosis* serotype 1 infection. The lesions of this disease may not be observed up to several months after initial infections. The disease is often referred to as a hidden disease and only becomes apparent during meat inspection [30], and it is manifested in two different forms: (external caseous lymphadenitis) abscesses in superficial lymph nodes and subcutaneous tissue; and (visceral caseous lymphadenitis) abscesses in internal organs [4, 36, 42]. The disease causes significant economic losses due to decreased meat production, damaged wool and leather, as well as decreased reproductive efficacy. Once the disease is established, it is difficult to be treated because antibiotic therapy is not effective. So, massive vaccination may be necessary to reduce the diseases prevalence. Killed *C. pseudotuberculosis* adjuvant vaccine has been used for prevention and control of this disease, but its efficacy is variable and the reports about protection rates mostly variable. It was suggested that efficacy of killed adjuvanted vaccines depends mainly on the used adjuvant which is safe for use and able to enhance the vaccine immunogenicity [11, 14, 15].

The present study was directed to develop different formulae of oil adjuvant vaccine from killed local isolated *C. pseudotuberculosis* field strain and evaluation of their potency in Guinea-pigs and sheep.

2. MATERIALS AND METHODS

2.1. *Animals*:

2.1.1. *Guinea pigs*:

Twenty five Guinea pigs weighed 300-450 g were divided into five groups, each of 5 animals. Four groups were used to test the potency of the prepared *C. pseudotuberculosis* vaccine formulae and the fifth group was used as control.

2.1.2. *Sheep*:

Fifteen native sheep of about 6-8 months of age were apparently healthy with no history of caseous lymphadenitis and kept under clinical observation for four months. All sheep were selected for the experiment had negative ELISA test. The 15 sheep were divided into 5 groups where each group contained 3 sheep. The first four groups received vaccination and the fifth group was kept as control.

2.2. *Bacteria*:

A local isolates of *C. pseudotuberculosis* was used to prepare different formulae of an inactivated vaccines.

2.3. *BCG vaccine*:

BCG vaccine produced by Staten's Serum Institute, Denmark as Batch No. 107043A. was supplied by Holding Company of Vaccine and Sera (VACSERA) Cairo, Egypt and used for vaccination of sheep intradermally as heterologous living vaccine using sheep dose of (4× dose of an infant) [6].

2.5. *Vaccine preparation*:

2.5.1. *Killing of Corynebacterium pseudotuberculosis field strain using formalin*:

Killing or inactivation of *C.* pseudo-tuberculosis using formalin was carried out as the description of Brown et al. [12] for 2 days.

2.5.2. *Killing Corynebacterium pseudotuberculosis using Binary ethyleneimine*:

Binary ethyleneimine solution was added to the bacterial suspension in 10%, 20%, 30% to get a final concentration of 0.01%, 0.02% and 0.03% and incubated at 37^{o} C and checked as possible every one hour, and the viability were tested every 24 hours on brain heart infusion agar plates. The excess of inactivation in case of formalin and binary ethyleneimine and absence of any other micro-organisms was neutralized by sodium bisulphate and sodium thiosulphate respectively according to Farid et al. [21].

2.5.3. *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. [33] and Brain heart infusion media according to Brown et al. [12] the Phospholipiase D exotoxin was inactivated by addition of formalin 0.1% to the concentrated culture filtrate containing Phospholipiase D exotoxin (50 ug / ml) then mixed well and leaved for 24 hours at room temperature. Absence of toxin activity in toxoid preparation had been demonstrated as described by Muckle and Gyles [34] 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.

2.5.4. *Addition of adjuvant*:
2.5.4.1. *Water in oil emulsion*:
Paraffin oil adjuvant vaccine was composed of water oil ratio of 30/70. The oil phase composed of paraffin oil and span 80 at ratio of 9:1 respectively. A water phase was composed of concentrated toxoid of culture filtrate containing the killed bacteria and Tween 80 as an emulsifier agent in a concentration of 3% according to Jansen [27]. Dose of vaccine is 3 ml for sheep.

2.5.4.2. *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 Iyer et al. [26]. Dose of sheep in this vaccine is 2ml.

2.6. *Evaluation of Corynebacterium pseudotuberculosis vaccine*:
It was performed according to Office International Des Epizooties [35] including purity**;** safety and potency tests.

2.7. *Animal vaccination*:
2.7.1. *Guinea pigs vaccination*:
Five groups (each group contained 5 animals) were managed as follow:
Group 1: vaccinated with 2 doses, (each of 0.5 ml) of binary killed bacterin+ toxoid + Montanide ISA 206 oil adjuvant with 15 days in between.
Group 2: vaccinated with 0.5 ml of formalin killed bacterin + toxoid + Montanide ISA 206 oil adjuvant, in 2 doses with 15 days apart.
Group 3: vaccinated with 2 doses of binary inactivated bacterin + toxoid + Paraffin oil adjuvant, each was 0.8 ml with 15 days apart.
Group 4: vaccinated with 2 doses of formalin killed bacterin + toxoid + Paraffin oil adjuvant, each was 0.8 ml with 15-days-apart. Each Guinea pig was vaccinated with 1/4 dose of sheep according to Cameron and Fuls [14].

2.7.2. *Sheep vaccination*:
Sheep were divided into five groups (three animals/ group) as follow:
Group 1: Vaccinated with 0.4ml of BCG containing 1-2 million organism injected intradermally 2 months pre-vaccination with 2ml containing 20 mg bacterin + 50 µg toxoid + Montanide ISA 206 oil injected S/C as the first dose, then boostered with the same dose 15 days apart.
Group 2: Vaccinated with BCG (1-2 million organism) intradermally using a single dose, simultaneously with the first dose of 2ml containing 50 µg toxoid + Montanide ISA 206 oil adjuvant injected S/C then boostered with the same dose 15 days apart.
Group 3: Vaccinated with 1st dose of 2ml containing 20 mg bacterin + 50 µg toxoid + Montanide ISA 206 oil adjuvant and boostered with the same dose 15 days apart.
Group 4: Vaccinated with 1st dose of 2ml containing 10 mg bacterin + 50 µg toxoid + Montanide ISA 206 oil adjuvant injected S/C and boostered 15 days apart (half the dose of bacterin in previous groups).
Group 5: was experimentally infected with the 4×10^6 colony forming unit of *C. pseudotuberculosis* field strain.

2.8. *Evaluation of protection efficiency of different prepared Corynebacterium pseudotuberculosis vaccine formulae*:
2.8.1. *Determination of the cellular immune response through lymphocyte proliferation assay*:
It was applied according to the method adopted by Lee [29].

2.8.2. *Determination of humoral immune response using ELISA*:

ELISA was carried out according to Menzies et al. [32].

2.8.3. Challenge test:
Evaluation of vaccine potency by challenge exposure test and necropsy examination of the tested animals was carried out according to Johnson et al. [28].

2.8.3.1. *Challenge test in Guinea pigs*:
All groups were challenged intradermally10 days after the 2nd dose of vaccination and observed for 15 days after challenge by using 0.5 ml of 1×10^6 colony forming unit of *C. pseudotuberculosis* field strain. Then all animals were sacrificed and examined postmortem. Pathogenicity test in Guinea pigs to determine the activity of the challenge agent was done according to Muckle and Gyles [34].

2.8.3.2. *Challenge test in 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).

2.8.3.3. *Necropsy examination of the challenged animals*:
Control and Vaccinated sheep were slaughtered and necropsied 150 days (5 months) after the challenge according to Paule et al. [37] where all organs and all detectable lymph nodes were macroscopically evaluated for presence of abscesses and examined as follow to register abscess formation.

2.9. *Statistical analysis*:
Statistical analysis using ANOVA test was applied on immunogenic results according to Snedecor and Cochran [40] to compare the efficiency of the prepared vaccines for G. pigs and sheep.

3. RESULTS AND DISCUSSION

The present work was mainly planned to prepare and evaluate *C. pseudotuberculosis* vaccine from the local field isolated strain. The yield of PLD toxin using brain heart infusion media was about 6 times more than that using MEM synthetic media (Table-1) in agreement with those obtained by Paule et al. [37] and Soheir [41]. 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 [2].
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 as shown in table (2). Using both inactivated bacterin in immunization of Guinea pigs indicated that the mean levels of lymphocyte proliferation response and the mean levels of anti-PLD IgG in the animals immunized with formalin killed bacteria were nearly similar to those of BEI killed bacteria immunized Guinea pigs in agreement with Hussein and Salwa [25].
Table (3) showed that the mean levels of cell mediated immune response of Guinea pigs vaccinated with *C. pseudotuberculosis* vaccine adjuvanted with paraffin oil were less than those observed in groups vaccinated with the vaccine adjuvant with Montanide ISA-206. There was no significant difference between the cellular immune response stimulated by the prepared vaccines. While significant differences were found between the vaccinated groups and non-vaccinated one confirming that the prepared vaccines are capable to induce cellular immune response which play a role in protection against the organism as facultative mononuclear phagocytes

intracellular pathogen as reported by Cameron et al. [15] and Youssef [43].

The humeral immune response of vaccinated Guinea pigs as detected by ELISA (Table-4) showed that the highest mean levels of anti-PLD IgG were observed in animals vaccinated with Bacterin + toxoid adjuvanted with Montanide ISA 206 adjuvant in agreement with Barnett et al. [8] who stated that Montanide ISA 206 adjuvant formulation induced an earlier and higher immune response. ANOVA test indicated that there was no significant difference between the humoral immune response of Guinea pigs groups stimulated by the four prepared vaccines in accordance with Cameron and Fuls [14] and Pepin et al [38] who noticed that antibodies against *C. pseudotuberculosis* exotoxin increased from post inoculation by the day 5 and reached a plateau on 21 day post inoculation.

Table 1 Amount of secreted PLD toxin in brain heart culture filtrates and MEM culture filtrates concentrated by freeze drying and Millipore ultra-filtration

Detected items	Amount of PLD toxin in			
	Batch-1 using freeze drying (lyophilization)		Batch-2 using ultrafiltration concentration system	
	MEM	Brain Heart	MEM	Brain Heart
% in lane	21.213%	44.68%	20.53%	40.53%
Total protein of concentrated culture filtrate	26 ug/ml	70 ug/ml	28 ug/ml	73 ug/ml
Amount of PLD in concentrated filtrate	5.51 ug/ml	31.27 ug/ml	5.75 ug/ml	29.58 ug/ml

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

Time in hours post starting of inactivation	Inactivation rate of *C. pseudotuberculosis* using different concentrations of Binary ethyleneimine (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 and - = No growth = Complete inactivation

Table 3 Mean optical density of cell mediated immune response in Guinea pigs vaccinated with the prepared *C. pseudotuberculosis* vaccine formulae of which the antigens and adjuvants had been altered

Different vaccinal formulas	Cellular Response Post Vaccination							
	First dose of vaccine				Time of the second vaccination	Second dose of vaccine		
	0 day	7 days	10 days	13 days		16 days	19 days	22 days	25 days
Toxoid + Formalized bacterium + Paraffin oil	0.063	0.42	0.335	0.301		0.475	0.675	0.581	0.406
Toxoid + Formalized bacterium + Montanide	0.049	0.53	0.421	0.331		0.580	0.860	0.591	0.511
Toxoid + Bacterium inactivated by BEI + Paraffin oil	0.073	0.412	0.391	0.305		0.550	0.660	0.412	0.365
Toxoid + Bacterium inactivated by BEI + Montanide	0.059	0.495	0.412	0.401		0.731	0.821	0.602	0.542
Non-vaccinated animals	0.069	0.054	0.068	0.053		0.062	0.059	0.065	0.060

Table 4 Mean optical density values of humoral immune response in Guinea pigs vaccinated with the prepared *C. pseudotuberculosis* vaccine formulae of which the antigens and adjuvants had been altered

Different vaccinal formulas	Humoral Response Post Vaccination							
	First dose of vaccine				Time of the second vaccination	Second dose of vaccine		
	0 day	7 days	10 days	13 days		16 days	19 days	22 days	25 days
Toxoid + Formalized bacterium + Paraffin oil	0.132	0.405	0.588	0.613		0.642	0.691	0.701	0.751
Toxoid + Formalized bacterium + Montanide	0.143	0.490	0.577	0.631		0.701	0.792	0.859	0.921
Toxoid + Bacterium inactivated by BEI + Paraffin oil	0.136	0.353	0.441	0.532		0.552	0.577	0.632	0.701
Toxoid + Bacterium inactivated by BEI + Montanide	0.123	0.460	0.501	0.581		0.691	0.731	0.804	0.891
Non-vaccinated animals	0.138	0.129	0.144	0.145		0.170	0.153	0.147	0.162

The results of post mortem examination of vaccinated and challenged Guinea pigs (Table 5) showed the mean number of abscesses in skeletal muscles; lymph nodes and internal organs. The numbers of infected sites varied to lesser extent between the groups vaccinated with the same type of adjuvant. Such variation in the control group was significantly different to those of vaccinated groups confirming that all of the prepared vaccine antigens assessed a significant level of protection against infection. Also, from the post mortem findings in Guinea pigs vaccinated groups, it is clear that the group vaccinated with formalized bacterin + PLD toxoid and adjuvanted with Montanide ISA 206 was the best formula to be used for sheep vaccination. These results come in agreement and supported by those of Brogden et al [11] and Hodgson et al. [24] who concluded that absolute immunity could not be established. The concentrated vaccine may prove to be effective; and adding of an adjuvant can increase humeral responses and induce delayed hypersensitivity. Non vaccinated sheep group exhibited differences in their clinical appearance or had a rise of rectal temperature. After booster vaccination, transient mild swelling was developed at the vaccination sites which subsided within 7-14 days except one animal in group-1 and two in group-3which showed moderate abscessation at the site of vaccine injection. These findings agree with Eman [20] who mentioned that vaccinated animals remained clinically healthy after the first vaccination; however, for a short period after the 2nd vaccination there was depression, febrile reaction and three of ten vaccinated animals showed transient fibrous swellings at the site of vaccination.

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 the different prepared *C. pseudotuberculosis* vaccine increased in the second week of vaccination and reached its peak by the 1st week post the second dose (3rd week post the first vaccination) as in table (6) indicating that BCG followed by 50µg toxoid with 20 mg formalized bacterin adjuvanted with Mantonid oil induced the highest optical density values of cell mediated immune response in vaccinated sheep (group-1) followed by that in group-3 (in sheep vaccinated with 50µg toxoid with 20 mg formalized bacterin adjuvanted with Mantonid oil) then group-4 (in sheep vaccinated with 50µg toxoid with 10 mg formalized bacterin adjuvanted with Mantonid oil) with the lowest optical density value in group-2 (in sheep

vaccinated with BCG with 50µg toxoid adjuvanted with Mantonid oil).

In regards to using BCG as non-specific cellular immunostimulant heterogenous vaccine, Cameron and Fatthj [13] reported that immunization with BCG alone had no protective effect against caseous lymphadenitis, in contrast with Barakat [5] who concluded that BCG can be used alone for vaccination against caseous lymphadenitis where it induced protection of 90% in lambs under natural condition of infection.

ELISA showed that group-3 (in sheep vaccinated with 50µg toxoid + 20 mg formalized bacterin adjuvanted with Mantonid oil) exhibited the highest optical density level of humoral immunes response showing optical density of 1.005 by the 1st week post the second vaccination (3rd week post the first vaccination).

Table 5 Post mortem findings of challenged Guinea pigs groups vaccinated with different vaccine formulae expressed as the mean number of abscesses

Vaccine formulas (Groups)	No. of Guinea pigs	Skeletal lymph nodes				Internal organs				Total No. of abscesses
		Prefemoral lymph node		Prescapular lymph node						
		Right	Left	Right	Left	Lungs	Liver	Spleen	Kidney	
Group (1) Formalized C. ovis bacteria+PLD toxoid adjuvanted with Montanide ISA206	1	+ve	-ve	-ve	-ve	-	++	-	-	11
	2	+ve	-ve	-ve	-ve	-	+	-	-	
	3	+ve	-ve	-ve	-ve	-	++	-	-	
	4	-ve	-ve	-ve	-ve	-	++	-	-	
	5	-ve	-ve	-ve	-ve	-	-	-	-	
Group (2) Formalized C. ovis bacteria+PLD toxoid adjuvanted with paraffin oil	1	+ve	+ve	+ve	+ve	+	++	++	-	28
	2	+ve	+ve	-ve	-ve	-	++	+	-	
	3	+ve	+ve	-ve	-ve	-	+	+	-	
	4	+ve	+ve	-ve	-ve	-	++	-	-	
	5	+ve	+ve	-ve	-ve	-	+	+	-	
Group (3) Binary inactivated C. ovis bacteria+PLD toxoid adjuvanted with Montanide ISA206	1	+ve	-ve	-ve	-ve	-	++	++	-	15
	2	+ve	-ve	-ve	-ve	-	-	-	-	
	3	+ve	-ve	-ve	-ve	-	+	++	-	
	4	+ve	-ve	-ve	-ve	-	++	+	-	
	5	+ve	-ve	-ve	-ve	-	-	-	-	
Group (4) Binary inactivated C. ovis bacteria+PLD toxoid adjuvanted with paraffin oil	1	+ve	+ve	-ve	-ve	-	-	+	+	26
	2	+ve	+ve	+ve	-ve	-	++	++	-	
	3	+ve	+ve	-ve	-ve	-	+++	++	-	
	4	+ve	+ve	+ve	-ve	++	-	-	-	
	5	+ve	+ve	+ve	-ve	-	-	-	-	
Control group of Guinea pigs	1	C	C	C	C	C	C	C	C	62
	2	+ve	+ve	+ve	+ve	++	+++ +++	+++	+	
	3	+ve	+ve	+ve	-ve	+++	+++ +++	+++ ++	-	
	4	+ve	+ve	+ve	+ve	++ ++	+++ ++	++ ++	+	
	5	+ve	+ve	+ve	-ve	-	5+	+++	+	

+ve: caseted lymph node, -ve : No gross lesions, -: No foci, +: One foci, and C: Congestion

Table 6 Mean of cell mediated immune response of sheep vaccinated with different type of *C. pseudotuberculosis* vaccine adjuvanted by Montanide ISA 206 expressed by differential optical density values

Vaccine formulates	Cellular Response Post Vaccination								
	0 day	1 week	2 weeks	The second dose of vaccination	3 weeks	4 weeks	5 weeks	6 weeks	45 days	45 days time of challenge
BCG then 50ug toxoid + 20 mg Formalized bacterium + Montanide	0.147	0.480	0.540		0.570	0.499	0.280	0.262	0.188	
BCG +50 ug Toxoid + Montanide	0.157	0.240	0.342		0.368	0.367	0.240	0.226	0.211	
50 ugToxoid + 20 mg formalized bacterium + Montanide	0.164	0.344	0.447		0.497	0.401	0.303	0.250	0.210	
50 ugToxoid + 10 mg formalized bacterium + Montanide	0.143	0.327	0.450		0.468	0.397	0.277	0.203	0.203	
Non vaccinated animals	0.156	0.160	0.164		0.130	0.164	0.141	0.160	0.146	

Group-4 (in sheep vaccinated with 50µg toxoid + 10 mg formalized bacterin adjuvanted with Mantonid oil) came in the second grade of the humoral immune response with ELISA optical density of 0.980 followed by that in group-2 (in sheep vaccinated with BCG + 50µg toxoid adjuvanted with Mantonid oil) with optical density of 0.940 followed by the lowest on (0.834) in group-1 (in sheep vaccinated with BCG followed by 50µg toxoid + 20 mg formalized bacterin adjuvanted with Mantonid oil). These results are demonstrated in table (7). All the obtained cellular and humeral immune responses were parallel and agree with what reported by Garg and Chandiramani [23] who concluded that immunity to sonicated *C. pseudotuberculosis* cells was primarily of humeral nature, though cellular response was also noted. In contrast, live cells evoked primarily a cellular response and the encountered humoral response was of lesser intensity. In addition Brogden et al. [9] determined the degree of protection induced in lambs by inactivated *C. pseudotuberculosis* whole cell (WC) and cell wall (CW) vaccines. Immunized lambs were challenged. They found that the highest serologic response measured by tube agglutination test was seen in lambs vaccinated with CW bacterin. On the other side, Eggleton et al. [18] concluded that there is a positive correlation between amount of *C. pseudotuber-culosis* toxoid administered and degree of protection. So, they suggested that anti-toxic immunity is the major factor in protection.

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 and intense inflammatory swelling had been recognized and pus formation at the injection site in all sheep. Similar findings were reported by Doaa [17] and Eman [20] who found that the dose of challenge was able to induce signs of infection.

All animals were slaughtered after 150 days (5 months) and the post mortem findings and its evaluation were tabulated in Table (7).

Table 7 Mean humoral immune response of sheep vaccinated with different type of *C. pseudotuberculosis* vaccine adjuvanted by Montanide ISA 206 expressed by optical density values of indirect ELISA

Vaccine formulates	Humoral Response Post Vaccination								
	0 day	1 week	2 weeks		3 weeks	4 weeks	5 weeks	6 weeks	45 days
BCG then after 2 Month 50ug PLD totoxoid + 20 mg Formalized bacterium + Montanide	0.1415	0.369	0.61666	The second dose of vaccination	0.834	1.0166	0.986	0.922	0.940
BCG + Toxoid + Montanide	0.135	0.460	0.703		0.940	0.966	0.925	0.812	0.850
50 ugToxoid + 20 mg formalized bacterium + Montanide	0.140	0.535	0.864		1.005	1.166	0.960	0.983	0.900
50 ugToxoid + 10 mg formalized bacterium + Montanide	0.150	0.415	0.840		0.980	1.036	0.985	0.921	0.920
Non vaccinated animals	0.180	0.179	0.206		0.236	0.220	0.230	0.230	0.197

45 day's time of challenge

The different vaccinated sheep groups showed protection percentages against experimental infection of 71.2-78% while the percent of infection in unvaccinated sheep (control group) was 91%. This indicates that the prepared vaccine formulae are safe and potent sufficient to protect sheep. Hodgson et al. [24] and Piontkowski and Shivers [39] revealed that the percent of infection in unvaccinated control group was 100%. Hodgson et al. [24] and Piontkowski and Shivers [39] reported that the usage of the cell wall antigen and exotoxin phospholipase-D (the two major virulence factors of *C. pseudo-tuberculosis*) will compact the infection

Vaccination of sheep with concentrated culture filtrate toxoid plus whole cell bacterin adjuvanted with Montanide ISA 206 (first and fourth groups) elucidated a protection percentage range from 73.4% to 75% while on application of living attenuated cellular immunostimulant BCG vaccine preceding the previous vaccine the protection percent increased to 78% which was in agreement with Garg and Chandiramani [23] and Johnson et al. [28] who found that attenuated strain of *C. pseudotuberculosis* elicited 95% protection and the concentrated culture supernatant associated with Freund's incomplete adjuvant (FIA) stimulated strong humoral response but without robust disease prevention.

Doaa [17] used a vaccine composed of recombinant mutant PLD toxoid plus formalized cells were treated by acetone and ethyl ether and adjuvanted with paraffin mineral oil elicited 62% protection.

Our study resulted in protection percentage reached 75% which may 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.

Table 8 Scores of lesions detected at post mortem examination of different challenged sheep groups:

Type of vaccine (Group No.)	Animal No.	External lymph nodes				Internal lymph nodes	Total score of infected lymph nodes	% of protection
		RPS	LPS	RPF	LPF			
Group (1) BCG, 2 months, bacterin+ 50µg toxoid +Montanide adjuvant	1	0/3	0/3	1/3	0/3	0/3	10/45	78 %
	2	0/3	0/3	2/3	0/3	0/3		
	3	0/3	0/3	1/3	0/3	3/3		
Group (2), BCG 1-2 million + 50µg toxoid + Montanide adjuvant	1	0/3	3/3	0/3	0/3	0/3	13/45	71.2 %
	2	0/3	0/3	1/3	0/3	2/3		
	3	3/3	0/3	1/3	0/3	3/3		
Group (3), Bacterin 20mg + 50 µg toxoid + Montanide	1	0/3	0/3	2/3	0/3	3/3	11/45	75 %
	2	0/3	0/3	0/3	0/3	2/3		
	3	0/3	0/3	1/3	0/3	3/3		
Group (4), Bacterin 10mg + 50µg toxoid + Montanide adjuvant	1	0/3	0/3	3/3	0/3	3/3	12/45	73.4 %
	2	0/3	0/3	2/3	0/3	0/3		
	3	0/3	1/3	0/3	0/3	3/3		
Group (5) Control Group	1	3/3	1/3	3/3	3/3	3/3	41/45	9 %
	2	3/3	2/3	3/3	3/3	3/3		
	3	3/3	2/3	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

This result agreed with Hodgson et al. [24] who had protection 44% with genetically inactivated phospholipase-D exotoxin and 95% protection by conventional formalin inactivated vaccine. In addition, the same theory of Eggleton et al. [19] and Fontaine et al. [22] was verified in our study, they stated that the toxoid vaccines prepared from *C. pseudotuberculosis* culture supernatant routinely contain other excreted secreted antigens, somatic and cell wall antigens which stimulate the protective immunity responses. Previous studies [1, 3, 7, 16] reported that usage of serial of oil adjuvants of Montanide series (viz Montanide ISA 25, 70, 57 and 206) resulted in enhancement of immune response and these new oil formulations have favorable characteristics of low viscosity, lower reactivity and high potency. It elicits both humoral and cellular responses.

In regards to group 3 which was vaccinated with BCG as a non-specific heterogenous vaccine simultaneously with concentrated culture filtrate toxoid adjuvanted with Montanide ISA 206 we present 71.2 protection percent. Meanwhile, Youssef [43] recorded lower protection (66%) when he used BCG dissolved in recombinant mutated phospholipase-D and was injected subcutaneously. This could be explained that he did not use oil adjuvant with this vaccine.

The last group (fourth) in our study was immunized with lower dose of bacterin (10 mg). This group was vaccinated with 50 µg phospholipase-D concentrated culture filtrate and 10 mg formalized bacteria adjuvanted with Montanide ISA 206 preceding 71.2 protection percent and there was no abscessation at vaccinal injection site that agree with Brogden et al. [10] who represented that the vaccines containing 10 mg of whole cell induced sterile abscesses detected at the vaccination site in the vaccinated lambs, 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.

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مجلة بنها للعلوم الطبية البيطرية
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محاولات تحضير و تقييم لقاحات ضد السل الكاذب فى الاغنام

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الملخص العربى

تم خلال هذه الدراسة تحضير لقاح زيتى ضد مرض السل الكاذب فى الأغنام وذلك من عترة الميكروب المعزولة محليا حيث تم تركيز رشيح الميكروب للحصول على 50 ميكروجرام فوسفوليباز– د لكل 1سم وذلك بطريقتى التجفيف بالتبريد والترشيح مع التركيز. ووجد أن كلا الطريقتين آمنة ولكن التركيز بالترشيح يمثل طريقة أسهل فى صناعة اللقاح. وقد تم تثبيط البكتيريا بالبيناري إثيلين أمين بتركيزات مختلفة لمقارنته بالميكروب المثبط بالفورمالين. ووجد أن كلاهما يصلح لعمل اللقاح بنفس الكفاءة والفورمالين أفضل عملياً لسهولته. هذا وقد تم عمل تحضير 4 لقاحات لتجربتها فى خنازير غينيا وهى: (أ) لقاح زيتى بارافينى مع البكتيريا المثبطة بالفورمالين بالإضافة الى توكسيد الفوسفوليباز، (ب) لقاح زيتى بارافينى مع البكتيريا المثبطة بالبيناري بالإضافة لتوكسيد الفوسفوليباز، (جـ) لقاح زيتى مونتانيد 206 مع بكتيريا مثبطة بالفورمالين + توكسيد الفوسفوليباز، و (د) لقاح زيتى مونتانيد 206 مع بكتيريا مثبطة بالبيناري + توكسيد الفوسفوليباز. كما أجريت اختبارات مناعية لخنازير غينيا لقياس تحفيز الخلايا الليمفاوية وقياس الأجسام المناعية باختبار الاليزا و بعمل تحليل إحصائى لنتائج الاختبارات المناعية وجد أن مجموعات اللقاحات الأربعة تعطى جميعاً نتائج جيدة حيث لا فرق جوهرى بينهم ولكن هناك فروق جوهرية مع المجموعة التى لم يتم تحصينها. وعند إجراء اختبار التحدى وتشريح خنازير غينيا وجد أن اللقاح الأفضل هو اللقاح زيتى مونتانيد 206 مع البكتيريا المثبطة بالفورمالين بالإضافة الى التوكسيد. كذلك تمت تجربة هذا اللقاح فى الأغنام بأربعة طرق مختلفة: (1) لقاح مكون من 50 ميكروجرام توكسيد الفوسفوليباز + 20 ملليجرام بكتيريا مثبطة + محفز مناعى مونتانيد 206.، (2) حقن لقاح بى سى جى فى أدمة الجلد كمحفز مناعى خلوى ثم حقن اللقاح المذكور فى (1) تحت الجلد بعدها بشهرين، (3) تم حقن لقاح ال بى سى جى بأدمة الجلد فى نفس الوقت مع لقاح السل الكاذب المكون من 50 ميكروجرام توكسيد الفوفسولييباز + المحفز المناعى مونتانيد 206 تحت الجلد، و (4) تم حقن لقاح مكون من 50 ميكروجرام توكسيد + 10 ملليجرام بكتريا مثبطة (نصف جرعة البكتريا فى المجموعتين الاوليين) + محفز مناعى مونتانيد 206. ثم تم عمل اختبارات مناعية للأغنام لقياس المناعة الخلوية عن طريق اختبار تحفيز الخلايا الليمفاوية والأجسام المناعية عن طريق اختبار الاليزا وعمل تحليل إحصائى للنتائج ووجد أن جميع اللقاحات تصلح للوقاية ضد المرض. كما تم إجراء اختبار التحدى لتقييم القدرة الوقائية للقاحات المختلفة وتم ذبح الأغنام وتشريحها بعد 5 شهور حيث تراوحت نسبة الوقاية للأغنام بين 78% – 71.2%.

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