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Evaluation of Protective Efficacy of Mixed PLD Toxoid and Clostridial Vaccines Against Caseous Lymphadenitis (CLA) in Small Ruminants at Egypt

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Abstract: The protective efficacy of two formulated vaccines against *Corynebacterium pseudotuberculosis* biotype 1 was tested on male sheep (Local sheep bread Balady) in a herd free from caseous lymphadenitis (CLA) using a virulent strain of *C. pseudotuberculosis* biotype 1 (nitrate negative), locally isolated from sheep severely infected with caseous lymphadenitis (CLA), the capacity of the two experimental vaccines to protect against experimental challenge was determined by the appearance of the manifestation of disease that were equivalent to the naturally observed infection in Egypt. The animals were divided into 3 groups, each one group of 3 animals was immunized with combined vaccine, the first vaccine composed of toxoid PLD plus Covaccine 8., second vaccine composed of toxoid PLD plus polyvalent clostridial vaccine locally produced and control groups of unvaccinated animals. All groups were challenged by 4×10^6 CFU forming unit per ml of live virulent strain of *Corynebacterium pseudotuberculosis* isolated from local sheep infected with caseous lymphadenitis. Unvaccinated animals showed manifestations of caseous lymphadenitis (CLA) that Cleary observed in naturally diseased animals, the humeral immune response was evaluated by ELISA . There was an increase of the titer of PLD antibodies and clostridial antitoxin titer. The degree of protection induced by vaccines was investigated by the post mortem finding of abscesses and/or bacteria, it was found that toxoid PLD plus Covaccine 8 vaccine provided protection (78%) against caseous lymphadenitis in sheep in compared with the other type of vaccine toxoid PLD plus polyvalent clostridial vaccine that gave only level of protection 64%. The present study indicated that combination of toxoid PLD and clostridial vaccine together can stimulate humeral immune response against the development of caseous lymphadenitis (CLA) as the combined vaccine succeeded in providing absolute protection against infection.

Key words: *Corynebacterium pseudotuberculosis* · Abscesses · Caseous lymphadenitis clostridial vaccine

INTRODUCTION

Caseous lymphadenitis is a worldwide disease caused by *Corynebacterium pseudotuberculosis* bio type 1 that causes significant economic losses to sheep and goat producers as it reduces milk yield, and meat& wool production. Also decreases reproductive efficiencies of affected animals [1, 2]. The most obvious frequent form of CLA in small ruminants is characterized by abscess formation in subcutaneous tissues and caseous superficial lymph nodes by production of caseation, necrosis at the lymphnode. There is another form of CLA called visceral form characterized by development of internal abscess in kidneys, lungs, spleen and liver [3].

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C. pseudotuberculosis produced a potent exotoxin Phospholipase D (PLD) that is considered the major protective antigen and the most important virulence factor for the bacterium [4]. PLD plays an important role in the dissemination of the bacteria from the site of infection to other sites in the host by enhance the hydrolysis of ester bonds in sphingomyelin of mammalian cell membranes [5-7] also PLD have a lethal effect as it causes destruction and damage of caprine macrophages [8]. The molecular structure of *C. pseudotuberculosis* PLD is present in the venom of the medically important spider genus *Loxosceles* its present in sphingomyelinases [7, 9, 10].

As a result of the characteristic immunogenic features of the PLD, it has often been used as a tool in the preparation of an efficacy protective vaccine against CLA. The vaccines that are currently produced for control of CLA generally use culture supernatants of *C. pseudotuberculosis* rich in formalin-inactivated PLD as toxoid vaccines that are obtained from PLD-rich *C. pseudotuberculosis* culture supernatant [11-16], Hodgson *et al*. [17] investigated the potency of formalin inactivated PLD toxoid vaccine and other one prepared by genetic inactivation of PLD gene that give protection 44% post challenge with virulent strain of *C. pseudotuberculosis*, while the other vaccine give 95% protection. Other researchers studied the effect of changing one or two amino acids at specific sites of PLD for preparation of toxoid inactivate vaccine as Selim *et al.* [18] who investigated the efficacy of genetically inactivated PLD expressed in *E.coli* to obtain amutated recombinant mrPLD combined with formalin killed whole *C. pseudotuberculosis* that give 78% protection. live vaccines, inactivated whole-cell vaccines and DNA-vaccines have also been investigated. Even though none of these vaccines provide an overall protection against CLA, they have been shown to limit the spread of the disease and significantly decrease the prevalence in the herd as well as reduce the clinical symptoms in vaccinated animals [19, 20]. So another formula of available commercial toxoid vaccine for caseous lymphadenitis has been investigated by combination of toxoid vaccines ýwith vaccines against other pathogens, these include *Clostridium perfringens*, *Cl. tetani*, *Cl. septicum*, *Cl. chauvoei* and *Cl. Novyi* [19, 21, 22], the researchers reported a decrease in in the spread of the disease in animals and also decrease the size and number of CLA lung abscesses.

The objective of the present study was directed to perform a study of the protective efficacy of two different toxoid vaccine formulation combined with Clostridial toxins (*Clostridium perfringens*, *Cl. tetani*, *Cl. septicum*, *Cl. chauvoei* and *Cl. Novyi)* to evoke protection against CLA and Clostridial diseases in sheep against challenge with *C. pseudotuberculosis* virulent strain locally isolated from sheep suffered from CLA in Egypt.

MATERIAL AND METHODS

Animals (Sheep): Nine sheep from Rasseder farm of Animal Health, Desert Research Center approximately 8–10 months old were tested by ELISA to exclude the positive reactors.

Vaccines:

Vaccine 1: Combined Vaccine of Toxoid PLD with Clostridial Vaccine (Covaccine 8) [13]: A previous preserved *C. pseudotuberculosis* sample from the previous study by Selim *et al*. [23], it was used a nearly passage of the most virulent strain isolated from sheep having caseous lymphadenitis (CLA) it was inoculated in Brain Heart Infusion agar (Oxoid) supplement with fosfomycin (Sigma) 200mg, Nalidixic acid 4mg /liter, incubated for 48 hour at 37°C, this isolate was used for preparation of phospholipase D (PLD gene) and challenge of sheep during experiments of vaccine potency measurement, the highly virulent strain of *C. pseudotuberculosis* it was completely identified biochemically and with PCR for detection of (PLD gene) as our previous published paper [23] also measurement of its haemolytic activity by modified CAMP test and reverse CAMP test as described and done by in our published paper [23]. Preparation of culture filtrate was made in two stages media as described by [24], the strain was inoculated in cooked meat media for 24 hours then transferred to brain heart infusion broth containing Tween 80 (1500 ml), incubated in shaking incubator at 37°C for 48 hour, the supernatants were collected and its total proteins was assayed by Lowry method [25], then it was concentrated in cooling concentrator then, the concentrate was electrophoresed by SDS-PAGE and analyzed by the gel pro computer software to determine the amount of PLD and bands were detected by immunoblot technique using anti PLD hyper immune serum prepared in the Biotechnology research center at veterinary medicine Cairo university [26].

PLD was inactivated by addition of formalin 0.1 % to 2000 ml culture filtrate then lyophilized and weight.as 2000 ml filtrate contained 64 gm of lyophilized each dose (2 ml) contained 23µg PLD, 40 ml of covaccine 8 (An imported vaccine formulated from a mixture of Clostridial toxins, obtained from Schering Plough Animal Health) were mixed with 6 gm of lyophilized powder of formulated culture of PLD.

Vaccine 2: Toxoid PLD Vaccine Combined with Clostridial Vaccine (Polyvalent Clostridial Vaccine): Prepared by Vet. Serum and Vaccine Research Institute, Abbasia; 40 ml of polyvalent Clostridial vaccine mixed with 6 gm lyophilized powder of formulated culture filtrate of PLD

Adjuvant Preparation [27]: Water in oil emulsion adjuvant used for preparation of vaccine was composed of water /oil ratio of 30/70, the oil composed of mineral oil and span 80 at ratio of 9:1 respectively, an emulsifier (Tween80) was used as surfactant at a concentration of 3%.

Vaccination and Experimental Challenge: Each vaccine was inoculated into a group of 3 animals distributed as.

Group D consisted of 3 animals numbered as D1, D2, & D3, each animal was inoculated subcutaneously by 2 ml of vaccine (Toxoid PLD with clostridial vaccine (Covaccine 8) in theleft of axillary region. The same dose was repeated after 4 weeks.

Group E consisted of 3 animals numbered as E1, E2, E3 each animal was inoculated subcutaneously by 2 ml of vaccine (Toxoid PLD +polyvalent clostridial vaccine) in the left of axillary region. The same dose was repeated after 4 weeks.

Group C constituted of 3 animals numbered as C1, C2 & C3, each animal was inoculated subcutaneously with 4ml saline adjuvant mixture.

Challenge exposure [4]. All vaccinated and control sheep were challenged with 5 ml of 48 h brain heart broth which contained 4×10^6 of living cells forming unit per ml, inoculated by S\C with 1 ml of diluted broth in the right axillary region four weeks after second dose of vaccination.

Scar Evaluation of the Developed Regions: Besides sites of challenge inoculation, the internal organ lymph nodes were examined for internal abscesses and samples were collected from abscesses for reisolation of *C. pseudotuberculosis*. Examined L.N were divided into 5 groups external right, left prescapular, right & left prefemoral and fifth group containing all of internal organs (Right & left popliteal, bronchial, thoracic, inguinal, mesenteric). Abscess in inoculation site was considered as one unit. Each group is considered in case of scar evaluation, scar of infection in each unit was calculated according to size, shape, appearance of lymph node.

The following score was used, enlarged and caseated L.N 3\3, enlarged, oedematous or congested L.N 2\3, enlarged L.N 1\3, normal L.N 0\3.

Therefore the score calculation of each group (composed of 3 animals) was done as follow:

Maximum score for each unit =3\3
Maximum score of the 3 animals =3×3=9
Maximum score of all units of L.N in each group =9×6=54

Serology: Animal's immunological responses to vaccination and /or infection were determined by measurement of serum anti-PLD IgG, clostridial antitoxin by ELISA by using mutated r PLD [26], Clostridial toxins antigen. Ninety-six well microtiter plates were coated with mutated r PLD and bound anti-PLD IgG was detected by the use of anti-bovine conjugated with alkaline phosphatase (Sigma) which developed a color by addition of P-nitrophenyl phosphate substrate-calorimetric reactions were stopped and intensified, reaction were stopped and intensified by addition of IN NaOH, (50: 1/well) and the absorbance of each sample at 450 mm was subsequently determined. All the previous procedure was repeated by use of the Clostridial toxins antigen.

RESULTS

Evaluation of the Productive Efficacy of Vaccines Formulations: After challenge all sheep were lethargic for the first 72 hours then return to normal temperature, there were no significant change except at the first day. a transient sterile nodule from 2 to 4 cm in diameter was seen at the inoculation site, and then disappeared after 2weeks. The evaluation of humeral immune response by direct enzyme linked immunosorbent assay (ELISA)showed that the mean optical density (OD) Values of antibody titer in sera of sheep vaccinated with toxoid PLD combined with Covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial vaccine (Group E), were higher than cut off value (Table 1,2 & Fig. 1). Statistical analysis using GLM model illustrated that there was a highly significant ($p<0.001$) difference among the mean OD values of antibody titer in sera of vaccinated and control sheep after 4 weeks from the first dose of vaccination, on the other hand there was no significant difference between the mean of clostridial antitoxins titer in sera of sheep vaccinated with toxoid PLD combined with covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial vaccine (Group E) (Table 3, Fig. 2).

Table 1: Mean OD± standard error of phospholipase –D antibody titer in sera of sheep before vaccination (Zero time)

Type of vaccine	Animal number	OD	Mean OD
Toxoid PLD plus Covaccine 8 (group D)	D1	0.169	0.157
	D2	0.143	
	D3	0.161	
Toxoid PLD plus polyvalent Clostridial vaccine (Group E)	E1	0.146	0.148
	E2	0.163	
	E3	0.135	
Control group (group C)	C1	0.161	0.164
	C2	0.167	
	C3	0.164	

Cut off value=0.328, the mean optical density of antibody titer in sera of sheep was less than cut off value and seronegative.

Table 2: Mean OD ± standard error of phospholipase –D antibody titer in sera of control and vaccinated sheep after 4 weeks from the first dose of vaccination

Type of vaccine	Animal number	OD	Mean OD	±SE
Toxoid PLD plus Covaccine 8 (group D)	D1	0.465	0.576	0.0723a
	D2	0.712		
	D3	0.552		
Toxoid PLD plus polyvalent Clostridial vaccine (Group E)	E1	0.488	0.540	0.0618a
	E2	0.664		
	E3	0.470		
Control group (group C)	C1	0.165	0.163	0.008b
	C2	0.162		
	C3	0.164		

OD: optical density of colored reaction. Cut off value was calculated as double mean of OD of control non vaccinated group, cut of value =0.327. SE; Standard error means with the same letter in the same column are not significantly different.

Table 3: Mean OD ± standard error of clostridial antitoxin titer in sera of control and vaccinated sheep after 4 weeks from the first dose of vaccination

Type of vaccine	Animal number	OD	Mean OD	±SE
Toxoid PLD plus Covaccine 8 (group D)	D1	0.471	0.524	0.0359
	D2	0.510		
	D3	0.593		
Toxoid PLD plus polyvalent Clostridial vaccine (Group E)	E1	0.426	0.417	0.0215
	E2	0.376		
	E3	0.449		
Control group (group C)	C1	0.142	0.164	0.0032
	C2	0.153		
	C3	0.145		

OD: optical density of colored reaction. Cut off value was calculated as double mean of OD of control non vaccinated group, cut of value =0.29. SE; Standard error

Humeral Immune Response after 4 Weeks from the Second Dose of Vaccination: The data in Table (4) showed that the mean optical density (OD) Values of antibody titer in sera of sheep vaccinated with toxoid PLD plus Covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Group E) were higher than the cut off value (Table 4, Fig. 1). Statistical analysis illustrated that there was highly significant ($p<0.001$) difference among the mean OD values of antibody titer in sera of vaccinated and control sheep after 4 weeks from the second dose of vaccination, regard ling the type of vaccine, the mean OD values of phospholipase –D antibody titer in sera of sheep vaccinated with toxoid PLD plus Covaccine 8 (Group D) and mean OD values of phospholipase –D antibody titer in sera of sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Group E) were not significant. On the other hand there was no significance difference between the mean OD values of the clostridial antitoxins titer in sera of sheep vaccinated with toxoid PLD combined with Covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Group E) (Table 5, Fig. 2).

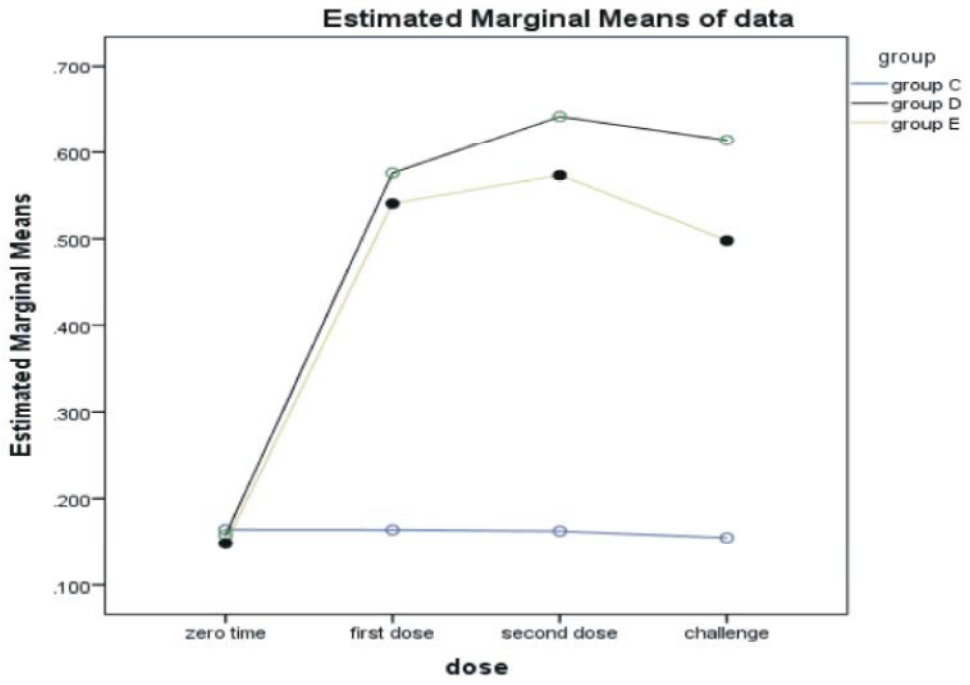

Fig. 1: Mean OD (Estimated marginal) of phospholipase –D antibody titer in sera of control and vaccinated sheep with Toxoid PLD plus Covaccine 8 (Group D) and with Toxoid PLD plus polyvalent Clostridial vaccine (Group E) collected at time intervals post immunization; C: Control group

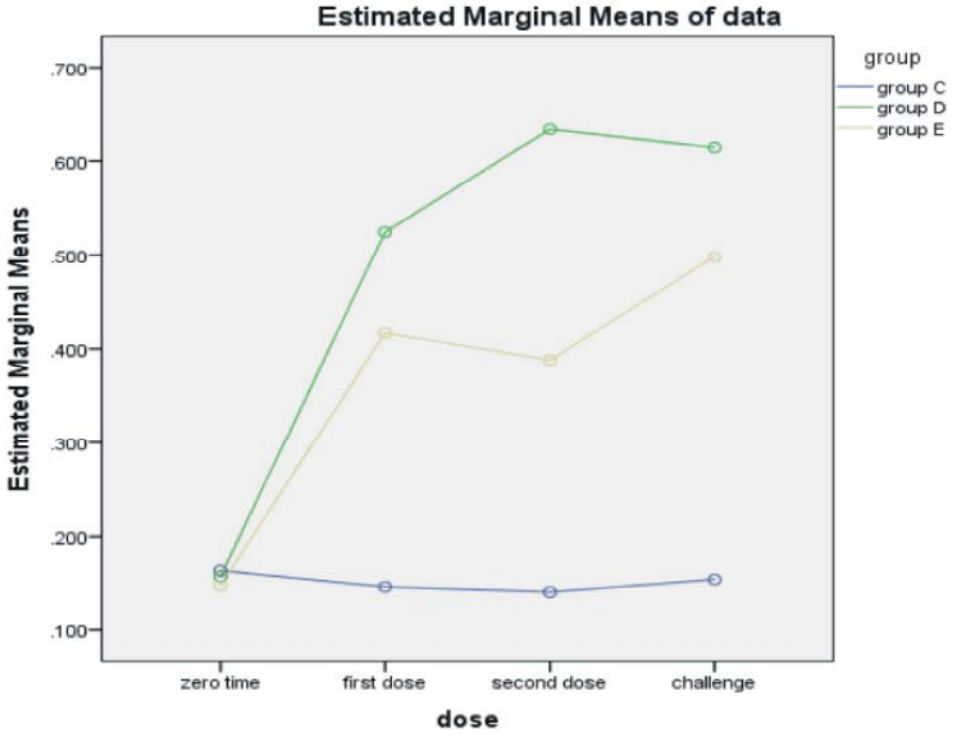

Fig. 2: Mean OD (Estimated marginal) of clostridial antitoxin titer in sera of control and vaccinated sheep with Toxoid PLD plus Covaccine 8 (Group D) and with Toxoid PLD plus polyvalent Clostridial vaccine (Group E) collected at time intervals post immunization; C: Control group

Table 4: Mean OD and ± standard error of phospholipase –D antibody titer in sera of control and vaccinated sheep after 4 weeks from the second dose of vaccination

Type of vaccine	Animal number	OD	Mean OD	±SE
Toxoid PLD plus Covaccine 8 (group D)	D1	0.534	0.641	0.0809a
	D2	0.800		
	D3	0.590		
Toxoid PLD plus polyvalent Clostridial vaccine (Group E)	E1	0.533	0.573	0.0541a
	E2	0.681		
	E3	0.507		
Control group (group C)	C1	0.165	0.162	0.0018b
	C2	0.159		
	C3	0.163		

OD: optical density of colored reaction.
Cut off value was calculated as double mean of OD of control non vaccinated group, cut of value = 0.324
SE; Standard error

Table 5: Mean OD and ± standard error of clostridial antitoxin titer in sera of control and vaccinated sheep after 4 weeks from the second dose of vaccination

Type of vaccine	Animal number	OD	Mean OD	±SE
Toxoid PLD plus Covaccine 8 (group D)	D1	0.636	0.634	0.0115
	D2	0.653		
	D3	0.613		
Toxoid PLD plus polyvalent Clostridial vaccine (Group E)	E1	0.484	0.388	0.0480
	E2	0.338		
	E3	0.342		
Control group (group C)	C1	0.133	0.141	0.0041
	C2	0.147		
	C3	0.143		

OD: optical density of colored reaction. Cut off value was calculated as double mean of OD of control non vaccinated group, cut of value =0.282. SE; Standard error

Table 6: Mean OD and ± standard error of phospholipase –D antibody titer in sera of control and vaccinated sheep After 4 weeks from challenge with virulent *C. pseudotuberculosis*

Type of vaccine	Animal number	OD	Mean OD	±SE
Toxoid PLD plus Covaccine 8 (group A)	D1	0.677	0.689	0.0876a
	D2	0.847		
	D3	0.544		
Toxoid PLD plus polyvalent Clostridial vaccine (Group B)	E1	0.573	0.570	0.0739a
	E2	0.441		
	E3	0.697		
Control group (group C)	C1	0.164	0.139	0.0035b
	C2	0.134		
	C3	0.138		

OD: optical density of colored reaction.
Cut off value was calculated as double mean of OD of control non vaccinated group, cut of value =0.278
SE; Standard error

Humeral Immune Response after 4 Weeks from Challenge with Virulent of *C. pseudotuberculosis*: The data in Table 6 & Fig. 1 showed that the mean optical density (OD) Values of antibody titer in sera of sheep vaccinated with toxoid PLD plus Covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Group E) were higher than the cut off. Statistical analysis illustrate that there was highly significant ($p<0.001$) difference among the mean OD values of antibody titer in sera of vaccinated and control sheep After 4 weeks from challenge, regard ling the type of vaccine, the mean OD values of phospholipase –D antibody titer in sera of sheep vaccinated with toxoid PLD plus Covaccine 8 (Group D) and mean OD values of phospholipase –D antibody titer in sera of sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Group E) were not significance. On the other hand there is no significance difference between the mean OD values of the clostridial antitoxins titer in sera of sheep vaccinated with toxoid PLD combined with Covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Groupe E) (Table 7, Fig. 2).

Table 7: Mean OD and ± standard error of clostridial antitoxin titer in sera of control and vaccinated sheep after 4 weeks from challenge with virulent *C. pseudotuberculosis*

Type of vaccine	Animal number	OD	Mean OD	±SE
Toxoid PLD plus Covaccine 8 (group D)	D1	0.685	0.614	0.0390
	D2	0.608		
	D3	0.550		
Toxoid PLD plus polyvalent Clostridial vaccine (Group E)	E1	0.488	0.464	0.0130
	E2	0.443		
	E3	0.563		
Control group (group C)	C1	0.139	0.139	0.0012
	C2	0.142		
	C3	0.183		

OD: optical density of colored reaction. Cut off value was calculated as double mean of OD of control non vaccinated group, cut of value =0.279. SE; Standard error.

Table 8: Analysis of variance showing the effect of different types of vaccine on titer of antibodies

S.O.V	D.F.	Mean Square of PLD	Mean Square of CL
Between the variation of O.D. of antibody titer in different types of vaccine	14	0.130**	0.069**
Error	28	0.0156	0.001

S.O.V.; Source of variance. O.D. Optical density. D.F. Degree of freedom
PLD; Phospholipase –D-antibody. CL :Clostridium antitoxin
**< 0.01; highly Significant

Table 9: The mean value of phospholipase –D- titer by using different types of vaccine

			PLD antibody		Clostridial antitoxin	
Type of vaccine	Period	N.	Mean	SE±	Mean	SE±
Control	FM	3	0.163	0.0080 b	0.146	0.0032 f
Toxoid PLD+Covaccine8(group D)	FM	3	0.576	0.0723a	0.524	0.0359 b
Toxoid PLD +P. Clostridial vaccine(Group E)	FM	3	0.540	0.0618a	0.417	0.0215 ed
Control	SM	3	0.162	0.0018b	0.141	0.0041 f
Toxoid PLD+Covaccine8(group D)	SM	3	0.641	0.0809a	0.634	0.0115 a
Toxoid PLD +P. Clostridial vaccine(Group E)	SM	3	0.573	0.0541a	0.388	0.0480 ed
Control	P. Chall	3	0.139	0.0035b	0.139	0.0012 f
Toxoid PLD+Covaccine8(group D)	P. Chall	3	0.689	0.0876 a	0.614	0.0390 a
Toxoid PLD +P. Clostridial vaccine(Group E)	P. Chall	3	0.570	0.0739a	0.464	0.0130 c

N; Number, ± SE; Standard error. P. Chall; Post challenge. FM; First dose of vaccination in first month. SM; Second dose of vaccination in second month.*Mean with the same letter in the same column are not significantly different

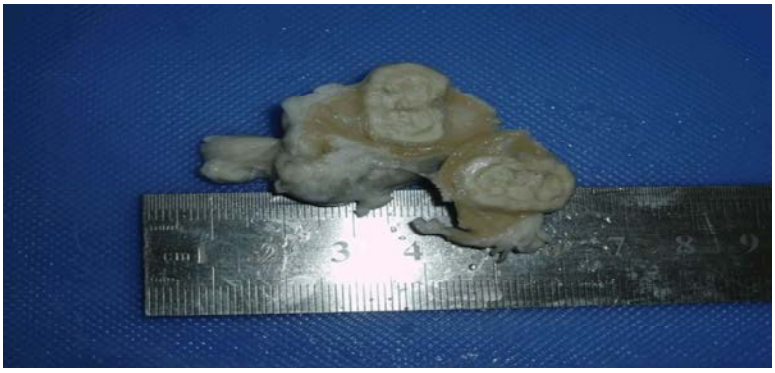

Fig. 3: Caseated (Prescapular) lymph node

The Post Mortem Findings of Sheep Vaccinated with Toxoid PLD Plus Covaccine 8: (Group D): This group included 3 sheep (D1, D2 & D3) as showen in Table (10). Post mortem finding of Animal number D1 showed that left prescapular lymph node enlarged and caseated as seen in Fig. 3, the right and left prefemoral were enlarged

Table 10: Scores of lesions detected during post mortem examination of external and internal lymph nodes of sheep post challenge

			External L.N							
Type of vaccine	Animal number	I.S	RPS	LPS	RPF	LPF	Total score of internal L.N	Total score of each group	% of infection	% of protection
Toxoid PLD+Covaccine 8 (group D)	D1	-0\3	-0\3	+++3\3	++2\3	++2\3	++2\3	12\54	22	78
	D2	-0\3	1\3+	-0\3	-0\3	-0\3	-0\3			
	D3	0\3-	-0\3	+1\3	-0\3	-0\3	-0\3			
Toxoid PLD+PolyvalentClostridial vaccine(group B)	E1	+1\3	++2\3	+++3\3	-0\3	++2\3	++2\3	13\36	36	64
	E2	Died	Died	Died	Died	Died	Died			
	E3	-0\3	-0\3	++2\3	-0\3	-0\3	-0\3			
Control unvaccinated (group C)	C1	3\3+++	+++3\3	+1\3	+++3\3	++2\3	+++3\3	40\54	74	26
	C2	+++3\3	+++3\3	++2\3	+++3\3	+1\3	++2\3			
	C3	++2\3	+++3\3	+1\3	+++3\3	-0\3	++2\3			

RPS: right prescapular lymph node RPF: right prefemoral lymph node

LPS: left prescapular lymph node LPF: left prefemoral lymph node

I.S: inoculation site of challenge Internal L.N: inguinal, tracheobronchial, thoracic, cervical and 2 popliteal lymph nodes

and oedematous. Also, the right popliteal lymph node was enlarged and oedematous. Animal number D2 showed that the right prescapular lymph node was enlarged and the left prefemoral were enlarged. Animal number D3 showed that the left prescapular lymph node was enlarged. The average of total score of lesions developed in all animals of this group was 12\54. This score revealed that the percent of infection 22% and protection was 78%.

The Post Mortem Findings of Sheep Vaccinated with Toxoid PLD plus Polyvalent Clostridial Vaccine: (Group E): This group included 3 sheep (E1, E2 & E3) as showen in Table 10, Postmortem findings of Animal number E1 showed swelling at site of inoculation, the right prescapular lymph node was enlarged and oedematous, the left prescapular lymph node was enlarged and caseated. On the other hand the left popliteal lymph node was enlarged and oedematous. Animal number E2 died after challenge due to urine retention as reveled by P.M lesion. Animal number E3 showed enlargement and oedematous left prefemorallymph node, the right prefemoral lymph node was enlarged and caseated. The average of total score of lesions developed in all animals of this group was 13\36. This score revealed that the percent of infection 36% and protection was 64%.

The Post Mortem Findings of Control Unvaccinated Sheep (Group C): This group included 3 sheep (C1, C2 & C3) as showen in Table 10 . All of which showed abscess at the inoculation site of living *C. pseudotuberculosis*. The right prescapular lymph node of all animals of this group showed caseation with typical onion like appearance of caseous lymphadenitis. The right prefemoral of all animals of this group were enlarged and caseated. The left prescapular lymph node was enlarged in animal number C1 while the left prefemoral was enlarged and oedematous. The left prescapular lymph node was enlarged and oedematous in animal number C2, the left prefemoral lymph node was enlarged in animal number C2. The left prescapular was enlarged in Animal number C3. On other hand, the right popliteal was enlarged and caseated in animal number C2, and enlarged & oedematous in animal number C3. The average total score of lesions developed in all animals of this group was 40\54. This score revealed that the percent of infection 74% and protection was 26%.

Bacteriological Examination of Infected Lymph Nodes and Abscesses in Inoculation Sites: *Corynebacterium pseudotuberculosis* was reisolated from all lymph nodes. Cross-sectional view of infected lymph node (Mediastinal) to CLA in the present study shows the calcium granules that deposited and had role to formation of lamellate in abscess.

DISCUSSION

The etiological agent of caseous lymphadenitis (CLA) or cheesy gland is *Corynebacterium pseudotuberculosis*, its presence in all goats and sheep production area causing great economic losses [19, 28]. The search of a formulation for production of an ideal vaccine against CLA, with less production costs and rare side effect was attracted many *C. pseudotuberculosis* researchers for induction of long acting and strong vaccine. Many different strategies have been studied including combination of supernatant and cellular components [29, 30], culture supernatants and cell-free toxoids [13, 31] bacterial cell-wall fractions, attenuated and killed bacteria [32, 33], DNA vaccines [34, 35] and recombinant proteins [6, 15, 17, 36, 37]. They investigated variable protection levels ranging from 38 to 98% against experimental and natural infections, but with undesirable safety profile owing to the presence of an abscesses at the site of inoculation site [16, 20 & 38-40] as Pépin *et al.* [41] who demonstrated that experimental infection with *C. pseudotuberculosis* in sheep can give protection

against further challenge but unable to clear the original infection to remain diseased carriers, to overcome this problems commercial vaccines for CLA are reported, these vaccines are formed of an inactivated PLD as it is the main component combined with other antigens from another pathogens as *Clostridium* [21, 22] that give significant protection against subsequent experimental infections with *C. pseudotuberculosis* [36], this combined vaccine was called (Glanvac TM) and became available in many countries [13]. Caseous D-TTM is another commercially available vaccine, it is formed of two component vaccine, which is a combination of *C. pseudotuberculosis* bacteria plus toxoid and one component vaccine that contains clostridial toxoids. The two-component vaccine have more protection against *C. pseudotuberculosis* infection than a one-component vaccine as it decreases the incidence of both internal and external CLA lesions [3]. PLD is an important virulence factor which have a great role in the dissemination of *C. pseudotuberculosis* from the primary site of infectionas as it impairs neutrophil chemotaxis toward the site of infection by increase vascular permeability so limiting bacterial opsonization [42]. The capacity of different forms of PLD-based vaccine to protect against CLA have been assessed by previous studies [13, 14, 17& 43]. Several authors have investigated that the protective immunity by vaccination with PLD derives from toxin neutralization as a result of action of anti-PLD antibodies [12, 14, 44, 45].

The role of various adjuvants in improving immune response to *C pseudotuberculosis* vaccine was examined by many investigators. Cameron & Bester studied the efficiency of different adjuvants that can be used during vaccine preparation[46], they tested the alum-precipitated vaccines, aluminum hydroxyl gel, aluminum ammonium sulphate, alum and sodium hydroxyl and saponin alum hydroxyl adjuvants. It was concluded that a combination of alum hydroxide and saponin provided better protection than vaccine only. Brodgen *et al*.used muramyl-dipeptide in association with 10% light mineral oil as adjuvant [47]. An emulsifier (Tween 80) was used as surfactant at a concentration 3% [43]. In our investigation we used oil adjuvant composed of mineral oil and spain 80 at a ratio of 9:1 respectively.

The present investigation was directed to evaluate the protective efficacy of toxoid PLD (Toxoid of *C. pseudotuberculosis*) combined with clostridial toxins (*Clostridium perfringens, Clostridium novyi, Clostridium tetani Clostridium septicum and Clostridium chauvoei*) as a polyvalent vaccine, the first vaccine used in this study was composed of toxoid PLD combined with clostridial vaccine (Covaccine 8) (Group D) prepared by addition of 40ml of Covaccine 8 obtained from Schering Plough Animal Health plus 6 gm lyophilized powder of culture filtrate of PLD. The second vaccine formulation composed of toxoid PLD vaccine combined with local polyvalent clostridial vaccine prepared by Vet. Serum and Vaccine Research Institute Abbasia, Egypt by adding 40ml of clostridial vaccine mixed with 6 gm lyophilized powder of culture filtrate of PLD (Group E). The efficacy of the different vaccine preparations was studied on group D of animals vaccinated subcutaneously with 2 ml of toxoid PLD combined with (Covaccine 8), group E of animals vaccinated subcutaneously with 2 ml of toxoid PLD vaccine combined with local polyvalent clostridial vaccine (Vaccine E) and (Group C) was used as negative control. All animals under experiment were subjected to challenge with a dose of 4×10^{6} CFU \ animal of living *C. pseudotuberculosis.* The same dose was used for experimental infection of sheep by Hodgson *et al*. [4] who found that the dose was able to induce signs of infection similar to natural infection in contrast to other researches [19, 30] who reported that four vaccinated groups of animals developed a robust humoral reaction against the bacteria one week after challenge with 1×10^{6} CFU of virulent *C. pseudotuberculosis*. All animals were lethargic for the first 72 after challenge then returned to normal temperature. This observation was recorded also by Piontkowski and Shivvers [3]. The result of humeral immune response was evaluated by ELISA, the results of antibody immune response of sheep group (D) and sheep group (E) showed higher titer of PLD antibody. There was no significant increase in the group mean antibody titers following primary, secondary vaccination and after 4 weeks from challenge as means of OD were 0.576, 0.641 and 0.689 respectively for PLD antibody in sheep group (D) vaccinated with toxoid PLD +Covaccine8 as seen in (Tables 2, 4, 6 & 9 and Fig. 1). On the other hand this group showed higher titer of clostridial antitoxins for the same vaccine (Mean OD 0.524, 0.634 and 0.614) respectively, while Mean OD for PLD antibody was (0.540, 0.573& 0.570) in sheep group (E) vaccinated with toxoid PLD +Polyvalent Clostridial vaccine and, means of OD were (0.417, 0.388 & 0.464) respectively for clostridial antitoxins as seen in (Tables 3, 5, 7 &9 and Fig. 2). The results indicated that the combination of toxoid PLD vaccine and clostridial vaccine together stimulated humeral immune response against caseous lymphadenitis and clostridial disease and this result coincides with Eggleton *et al.* [13].

The post mortem findings of sheep vaccinated with with toxoid PLD+Covaccine8 vaccine D revealed that the average total score of lesions developed in all animals of

this group was 12\45. This score revealed that the percent of infection was 22% and protection was 78%, while the percent of infection was 36%and protection was 64% for sheep vaccinated with Toxoid PLD+Polyvalent Clostridial vaccine as seen in Table 10. This results agrees with the data of Piontkowski and Shivvers [3] who reported that combined toxoid decreased the prevalence and number of abscesses that form secondary to *C pseudotuberculosis* infection, vaccinated sheep had significantly less external, internal, and total abscesses than control sheep. By comparing the results of vaccination provided by animals vaccinated with toxoid PLD plus Covaccine 8 (Group D) and sheep vaccinated with toxoid PLD plus polyvalent Clostridial (Group E) it was found that there was no significant difference in the level of anti PLD antitoxin or antibodies against local Clostridial strains could be observed. These results justified the possibility of a combined PLD toxoid-Clostridial vaccine by using locally prepared Clostridial vaccine. In conclusion mixed vaccines composed of toxoid PLD and clostridial vaccine have shown to induce protective immunity in sheep against 4×10^6 CFU of *C. pseudotuberculosis* from a virulent local strain. It will be necessary to demonstrate the role of cell mediated immunity for the same vaccine at the future.

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REFERENCES

1. Arsenault, J., C. Girard, P. Dubreuil, D. Daignault, J.R. Galarneau, J. Boisclair, C. Simard and D. Bélanger, 2003. Prevalence of and carcass condemnation from maedi-visna, paratuberculosis and caseous lymphadenitis in culled sheep from Quebec, Canada. Prev. Vet. Med., 59: 67-81.
2. Paton, M.W., I.R. Rose, R.A. Hart, S.S. Sutherland, A.R. Mercy, T.M. Ellis and J.A. Dhaliwal, 1994. New infection with *Corynebacterium pseudotuberculosis* reduces wool production. Aust.Vet. J., 71: 47-49
3. Piontkowski, M.D. and D.W. Shivvers, 1998. Evaluation of a commercially available vaccine *against Corynebacterium pseudotuberculosis* for use in sheep, J. Am. Vet. Med. Assoc, 212: 1765-1768.
4. Hodgson, A.L.M., J. Krywult, L.A. Corner, J.S. Rothel and A.J. Radford, 1992. Rational attenuation of *Corynebacterium pseudotuberculosis*: potential cheesy gland vaccine and live delivery vehicle, Infect. Immun, 60: 2900-2905.
5. Peel, M.M., G.G. Palmer, A.M. Stacpoole and T.G. Kerr, 1997. Human lymphadenitis due to *Corynebacterium pseudotuberculosis*: report of ten cases from Australia and review, Clin. Infect. Dis., 24: 185-191.
6. Tachedjian, M., J. Krywult, R.J. Moore and A.L. Hodgson, 1995. Caseous lymphadenitis vaccine development: site-specific inactivation of the *Corynebacterium pseudotuberculosis* phospholipase D gene, Vaccine, 13: 1785-1792.
7. Tambourgi, D.V., M.S. Da Silva, S.J. Billington, R.M. Gonçalves De Andrade, F.C.Magnoli, J.G. Songer and C.W. Van Den Berg, 2002. Mechanism of induction of complement susceptibility of erythrocytes by spider and bacterial sphingomyelinases, Immunology, 107: 93-101.
8. Tashjian, J.J. and S.G. Campbell, 1983. Interaction between caprine macrophages and *Corynebacterium pseudotuberculosis*: an electron microscopy study, Am. J. Vet. Res., 44: 690-693.
9. Binford, G.J., M.H.J. Cordes and M.A. Wells, 2005. Sphingomyelinase D from venoms of Loxosceles spiders: evolutionary insights from cDNA sequences and gene structure, Toxicon, 45: 547-560.
10. Van Meeteren, L.A., F. Frederiks, B.N.G. Giepmans, M.F.F. Pedrosa, S.J. Billington, B.H. Jost, D.V. Tambourgi and W.H. Moolenaar, 2004. Spider and bacterial sphingomyelinases D target cellular lysophosphatidic acid receptors by hydrolyzing lysophosphatidylcholine, J. Biol. Chem., 279: 10833-10836.
11. Jolly, R.D., 1965. The pathogenic action of the exotoxin of *Corynebacterium ovis*. J Comp Pathol., 75: 417-31.
12. Burrell, D.H., 1978. Vaccination against caseous lymphadenitis in sheep. In: The Proceedings of the 55th Annual Conference. Aust. Vet. Assoc., pp: 79-81.
13. Eggleton, D.G., C.V. Doidge, H.D. Middleton and D.W. Minty, 1991. Immunisation against ovine caseous lymphadenitis: efficacy of momocomponent *Corynebacterium pseudotuberculosis* toxoid vaccine and combined clostridial-corynebacterial vaccines. Aust. Vet. J., 68: 320-1.

14. Eggleton, D.G., J.A. Haynes, H.D. Middleton and J.C. Cox, 2005. Immunisation against ovinecaseous lymphadentitis:correlation between *Corynebacterium pseudotuberculosis* toxoid content and protective efficacy in combined clostridial-corynebacterial vaccines. Aust Vet. J., 68: 322-5.
15. Ribeiro, D., F.S. Rocha, K.M. Leite S.C. Soares, A. Silva, R.W. Portela, R. Meyer, A. Miyoshi, S.C. Oliveira, V. Azevedo and F.A. Dorella, 2014. An iron-acquisition-deficient mutant of *Corynebacterium pseudotuberculosis* efficiently protects mice against challenge. Vet. Res., 45: 28. doi:10.1186/1297-9716-45-28.
16. AdzaRina, M.N., M. Zamri-Saad, F.F.A. Jesse, A.A. Saharee, A.W. Haron and S. Shahirudin, 2013. "Clinical and pathological changes in goats inoculated *Corynebacterium pseudotuberculosis* by intradermal, intranasal and oral routes, "Journal of Veterinary Research, 17(2): 73-81.
17. Hodgson, A.L.M., K. Carter, M. Tachedjian, J. Krywult, L.A. Corner, M. McColl and A. Cameron, 1999. Efficacy of an ovine caseous lymphadenitis vaccine formulated using agenetically inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D. Vaccine, 17: 802-808.
18. Selim, S.A., M.E. Ghoniem and F.M. Khafaga, 2010. Vaccinal efficacy of genetically inactivated phosoholipase D agent against caseous lymphadenitis in small ruminant. International Journal of Microbiological Research, 1(3): 129-136.
19. Williamson, L.H., 2001. Caseous lymphadenitis in small ruminants. Veterinary Clinics of North America, Food Animal Practice, 17: 359-371.
20. Baird, G.J. and M.C. Fontaine, 2007. *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. J. Comp. Pathol., 137: 179-210.
21. Paton, M.W., S.B. Walker, I.R. Rose and G.F. Watt, 2003. Prevalence of caseous lymphadenitis and usage of caseous lymphadenitis vaccines in sheep flocks, Aust. Vet. J., 81: 91-95.
22. Stanford, K., K.A. Brogden, L.A. McClelland, G.C. Kozub and F. Audibert, 1998. The incidence of caseous lymphadenitis in Alberta sheep and assessment of impact by vaccination with commercial and experimental vaccines, Can. J. Vet. Res., 62: 38-43.
23. Selim, S.A., W.M. Mousa, K.F. Mohamed and I.M. Moussa, 2012. Synergistic haemolytic activity and its correlation to phospholipase D productivity by *Corynebacterium pseudotuberculosis* Egyptian isolates from sheep and buffalo. Brazilian Journal of Microbiology, pp: 552-559.
24. Syame Soheir, M., 2006. Characterization of secretory proteins that secreted from *Corynebacterium pseudotuberculosis*, Giza, Egypt, (Ph. D. Thesis. Bacteriology, Fac. Vet. Med., Cairo Univ.), pp: 150.
25. Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randali, 1951. Protein measurement with Folin-phenol reagent. J. Biol. Chem, 193: 265-275.
26. Ghoneim, M.A., A.W. Mousa, A.K. Ibrahim, A.S. Amin, A. Khafagi and S.A. Selim, 2001. Role of Hippobosca eqiunaas a transmitter of *C. pseudotuberculosis* among buffaloes as revealed by PCR and dot blothybridization.J. Egypt Vet. Med. Ass., 61: 165-176.
27. Jansen, T., M.P.M. H0fmans, M.J.G. Theelen and V.E.J.C. Schijns, 2005. Structure-activity relations of water in oil vaccine formulations and induced antigen -specific antibody responses Vaccine, 23: 1053-1060.
28. Paton, M.W., S.B. Walker, I.R. Rose and G.F. Watt, 2003. Prevalence of caseous lymphadenitis and usage of caseous lymphadenitis vaccines in sheep flocks, Aust. Vet. J., 81: 91-95.
29. El-Enbaawy, M.I., M.M. Saad and SA. Selim, 2005. Humoral and cellular immune responses of amurinemodel against *Corynebacterium pseudotuberculosis* antigens. Egypt. J. Immunol., 12: 13-20.
30. Braga, W.U., 2007. Protection in alpacas against *Corynebacterium pseudotuberculosis* using different bacterial components. Vet. Microbiol., 119: 297-303.
31. Mahmood, Z.K.H., F.F. Jesse, A.A. Saharee, S. Jasni, R. Yusoff and H. Wahid, 2015. "Clinio-pathological changes in goats challenged with *Corynebacterium peudotuberculosis* and its exotoxin (PLD), "American Journal of Animal and Veterinary Sci., 10: 112-132.
32. Simmons, C.P., A.L. Hodgson and R.A. Strugnell, 1997. Attenuation and vaccine potential of aro Q mutants of Corynebacteriumpseudotuberculosis. Infect. Immun, 65: 3048-3056.
33. Simmons, C.P., S.J. Dunstan, M. Tachedjian, J. Krywult, A.L.M. Hodgson and RA. Strugnell, 1998. Vaccine potential of attenuated mutants of *Corynebacterium pseudotuberculosis* in sheep. Infect. Immun, 66: 474-479.
34. Chaplin, P.J., R. De Rose and J.S. Boyle, 1999. Targeting improves the efficacy of a DNAvaccine against *Corynebacterium pseudotuberculosis* in sheep. Infect. Immun, 67: 6434-6438.

35. De Rose, R., J. Tennent and P. McWaters, 2002. Efficacy of DNA vaccination by differentroutes of immunisation in sheep. Vet. Immunol. Immunopathol., 90: 55-63.
36. Fontaine, M.C., G. Baird and K.M. Connor, 2006. Vaccination confers significant protection of sheep against infection with a virulent United Kingdom strain of *Corynebacterium pseudotuberculosis*. Vaccine, 24: 5986-5996.
37. Moore, R.J., L. Rothel, J. Krywult, A.J. Radford, K.L. Lund and A.L.M. Hodgson, 2000. Foreign gene expression in *Corynebacterium pseudotuberculosis*: development of a live vaccine vector. Vaccine, 18: 487-497.
38. Braithwaite, C.E., E.E. Smith, J.G. Songer and A.H. Reine, 1993. Characterization of detergent soluble proteins of *Corynebacterium pseudotuberculosis*. Vet. Microbiol., 38: 59-70.
39. Hodgson, A.L.M., M. Tachedjian, L.A. Corner and AJ. Radford, 1994. Protection of sheep against caseous lymphadenitis by use of a single oral dose of live recombinant *Corynebacterium pseudotuberculosis*. Infect. Immun, 62: 5275-5280.
40. Wilson, M.J., M.R. Brandon and J. Walker, 1995. Molecular and biochemical characterization of a protective 40 kilodalton antigen from *Corynebacterium pseudotuberculosis*. Infect. Immun, 63: 206-211.
41. Pépin, M., P. Pardon, J. Marly, F. Lantier and J.L. Arrigo, 1993. Acquired immunity after primary caseous lymphadenitis in sheep. Am. J. Vet. Res., 54: 873-877.
42. Carne, H.R. and E.O. Onon, 1978. Action of *Corynebacterium ovis* exotoxin on endothelial cells of blood vessels. Nature, 271: 246-8.
43. Brown, C.C., H.J. Olander, E.L. Biberstein and S.M. Morse, 1986. Use of a toxoid vaccine to protect goats against intradermal challenge exposure to *Corynebacterium pseudotuberculosis*. Am. J. Vet. Res., 47: 1116 9.
44. Batey, R.G., 1986. Pathogenesis of caseous lymphadenitis in sheep and goats. Aust Vet. J., 63: 269-72.
45. Sutherland, S.S., T.M. Ellis, M.J. Paton and A.R. Mercy, 1992. Serological response of vaccinated sheep after challenge with *Corynebacterium pseudotuberculosis*. Aust Vet. J., 69: 168-9.
46. Cameron, C.M. and F.J. Bester, 1984. An improved *Corynebacterium pseudotuberculosis* vaccine for sheep.Onderstepoort J. Vet. Res., 51(4): 263-267.
47. Brogden, K.A., J.S. Glenn, N. East and F.A. Audibert, 1996. *Corynebacterium pseudotuberculosis* bacterin with muramyl dipeptide induces antibody titers, increases the time of onset, and decreases naturally occurring external abscesses in sheep & goats. Small Rumin. Res., 19: 161-168.