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COMPARISON OF PROTECTION AGAINST CASEOUS LYMPHADENITIS IN SHEEP INDUCED BY LOCAL ISOLATED STRAIN OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* BY TOXOID PLD & TOXOID PLD WITH BACTERIN VACCINE

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ABSTRACT

Background: The objective of the present study was directed to perform a comparative study for the protective efficacy of different vaccine formulation to evoke protection against caseous lymphadenitis in sheep.

Materials and Methods: The protective efficacy of two formulated vaccines against Corynebacterium pseudotuberculosis biotype 1 was tested on 9 male local sheep bread (Balady) from a herd free from caseous lymphadenitis Disease. Using a virulent strain of C. pseudotuberculosis biotype 1 (nitrate negative), locally isolated from severely infected sheep with caseous lymphadenitis, all isolates were identified by standard microbiological techniques and by polymerase chain reaction targeting phospholipase D genes. Synergistic haemolysis of all isolates were assayed by modified CAMP test and reverse CAMP test. The presences of phospholipase D gene in supernatants of all isolates were performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoblot technique by using hyperimmune serum raised in rabbit immunized with recombinant phospholipase D gene antigen. The animals were divided into 3 groups each of 3 animals. Group A was immunized with Toxoid PLD, while group B was immunized with Toxoid PLD with Bacterine (formaline killed bacteria). Group C consisted of unvaccinated animals (control). All groups were injected by 2 doses of vaccine with 4 weeks interval then challenged four weeks after second dose of vaccination by 4 ×106 CFU forming unit per ml of live local isolated bacteria.

Results: Results showed. Unvaccinated animals showed manifestations of caseous lymphadenitis observed in naturally diseased animals .It was observed that antibody titer of sheep vaccinated with PLD vaccine showed the highest titer of PLD antibody and provide high level of protection against caseous lymphadenitis (91%) in compare with the other type of vaccine used (Toxoid PLD+Bacterine) as it give only level of protection 75%.

Conclusions: These results confirm the importance of PLD as a protective antigen and demonstrate the potential for developing caseous lymphadenitis vaccine.

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INTRODUCTION

C. Pseudotuberculosis is the pathogen of different disease in different animals, it is Gram-positive, non-spore forming; facultative anaerobic intracellular bacterium and catalase-positive, nitrate-reducing activity, its classified into 2 serotype and 2 biovar ; biovar (serotype 1) and biovar (serotype 2) (Barakat, 1984 and Baird, 2007). Biovar1 (serotype 1) (nitrate

negative) is the a causative agent of caseous lymphadenitis (CLA) in sheep and goat, a disease characteristic by loss of overlying hair enlargement, chronic absessation ,inflammation of one or more of the lymph node leads to granulomatous, necrotizing of it and finally rupture of the abscesses and discharge of pus (Williamson, 2001). The eradication of the disease is very difficult to the sheep and goat industry due to the lack of protective vaccines, limited reliability of diagnostic methods, its ability to persist in the environment and its poor response to therapeutics (Williamson, 2001). The major virulence factor expressed by *C. pseudotuberculosis* strains is the Phospholipase D (PLD) exotoxin (Hodgson, 1992 and Dorella, 2005). (PLD) is a potent secreted exotoxin have sphingomyelinase activity so increase vascular permeability in vivo in the presence of products from *Rhodococcus equi* (Batey, 1986 and Yozwiak, 1993) that have shown been a synergistic haemolysis (SH) of sheep blood cells and decrease the viability of ovine neutrophilis (Dorella, 2005). Subsequently many trails have been made for production effective CLA vaccines based on this antigen. The coat of waxy mycolic acids on the cell surface of *C. pseudotuberculosis* plays another major role in its pathogenesis, it supported the organism with mechanical and possibly biochemical protection from the hydrolytic enzymes in the lysosome to survive phagocytosis for extended periods within the environment as a facultative intracellular parasite (Baird, 2007; Williamson, 2001 and Brown, 1987).

Many attempts have been undertaken to develop a protective vaccine against CLA, none of these vaccines provide an overall protection against CLA as its only reduce the clinical symptoms in vaccinated animals by limiting the spread of the disease significantly and decrease its prevalence in the herd (Baird, 2007 and Williamson, 2001). Most of studies have investigated formalin-inactivated toxoid vaccines derived from PLD-rich *C. pseudotuberculosis* culture supernatants that have varying levels of protective immunity in both sheep and goats (Jolly, 1965; Nairn, 1977 and Selim, 2010), others investigated vaccination with killed whole-cells was sufficient to prevent the spread of *C. pseudotuberculosis* beyond the site of inoculation (Fontaine, 2006). For many years the potential to vaccinate sheep against infection with *C.pseudotuberculosis* with Bacterin vaccines have been shown a degree of protection against experimental infection (Brogden, 1984). This vaccine (Bacterin vaccines) also shown some success when used to vaccinate against naturally acquired infection in sheep and goats (Brogden, 1995 and Menzies, 1991), the use of cell wall and toxoid vaccines has been well investigated by many authors (Brogden, 1991; Paton, 1991; Paton, 2003; Braga, 2007; Brogden, 1996). The present study was performed to investigate the protective efficacy with 2 vaccine preparation. The first vaccine was formulated from Toxoid PLD, the second vaccine is fromToxoid PLD with Bacterine (formaline killed bacteria),these two vaccines was used to protect sheep against challenge virulent strain locally isolated from sheep have caseous lymphadenitis (CLA).

MATERIALS AND METHODS

Media and culture conditions

Aspirate pus(40) was collected from closed abscessed lymph nodes ,from periphery lesions of diseased animals , swabs was inoculated in Brain Heart Infusion agar (Oxoid,)supplement with fosfomycin (sigma)200mg, Nalidixic acid 4mg /liter, incubated for 48 hour 37Cº.

Biochemical identification of the C .pseudotuberculosis isolates

Local strains were completely identified by colonial characters, microscopic examination, different biochemical identification as (catalase test,urease hydrolysis test, nitrate deduction test, carbohydrate fermentation test, gelatin liquefaction test), measurement of haemolytic activity by modified CAMP test as described by (Bernheimer, 1980), and reverse CAMP test as described by.

Primers and PCR conditions for molecular identification of C .pseudotuberculosis isolates

The oligonucleotide primers used in this study were designed to detect PLD genes of *C. pseudotuberculosis*. The oligonucleotide primers specific for PLD genes of *C. pseudotuberculosis*; PLD F5': CGG CCC GGG ATT ATG GCG ATC ATG CTT C3' and PLD R5': CGC AAG CTT TCA CCA CGG GTT ATC CGC T 3' could amplify 930 base pair fragments. The PCR reactions was carry out according to Cetinkaya *et al* (Cetinkaya, 2002).

Characterization of phospholipase –D (PLD) by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot technique

Total proteins in each culture filtrate were measured by Lowry *et al*. (1951), then concentrated to 1/20 of the original volume (1 ml to 50 µl) by using the dry vacuum concentration (Speedvac System-Savant # SS11). Each concentration sample was treated with reducing buffer (Tris 91 g, SDS 1%, distilled water 500 ml) in the ratio of 1:1; the treated samples were immersed in a boiling water bath for 2 minutes to ensure protein denaturation. Electrophoresis was performed as described by (Laemmli, 1970), briefly 10 µl of each treated concentrate were loaded into each lane and electrophoresis was done for 4 to 6 hours at 100 vol and analyzed by the gel pro computer software to determine the amount of PLD. Proteins in unstained gels were electrotransferred to nitrocellulose membranes (Towbin, 1979). Membranes were blocked with blocking buffer (5% bovine serum albumin in 0.3% PBS-Tween, pH 7.2), washed in washing buffer and spliced into strips. In this investigation, we used highly purified recombinant PLD produced in BCVRS by Ghoneim *et al*. (Ghoneim, 2001), this recombinant PLD (rPLD) antigen was used for preparation of highly specific hyperimmune serum against PLD.The strips were exposed to diluted rabbit hyperimmune serum (1:50) and incubated for 1 hour at 25°C, washed 3 times (5 minutes each) in washing buffer and exposed to goat antirabbit IgG peroxidase conjugate (Sigma) diluted 1:1000. Bound antibody was visualized by use of 4-chloro-1 naphthol\H2O2 (0.5 mg /ml/0.15% in PBS with 17% methanol) as substrate. The quantity of PLD protein in each strain was analyzed using the gel pro software.

Animals: Nine male local sheep bread (Balady) animals were obtained from a herd with no previous history of caseus lymphadinities after examination their blood with Enzyme Linked Immunosorbent Assay(ELISA).

***Adjuvant preparation* (Jansen, 2005):** 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 (Tween 80) was used as surfactant at a concentration of 3%.

Vaccines

Vaccine 1: Toxoid culture filterate of C. Pseudotuberculosis (Brown *et al*., 1986)

Preperation of culture filterate from isolated strain was made in two stages media as described previous by Sohier. (Soheir, 2006), 2000 ml filterate was contain 64 gm of lyophilized powder, 12 ml of sterile distilled water were put to 6 gm of lyophilized powder to get water phase ,Then 25 ml mineral oil with 2.8 ml spain oil to get oil phase, finally 40 ml of vaccine contain 20 dose, each dose (2 ml) contained 23 mg PLD as described previous by Selim *et al*., (*Selim, 2016*).

Vaccine 2 : Formaline –killed C .pseudotuberculosis with toxoid PLD vaccine (Toxoid +Bacterine)

One well identified colony *of C .pseudotuberculosis* isolates were inoculated in 250 ml brain heart infusion broth containing 0.1 Tween 80 and incubated at 37°c for 24 hours with shaking .Then centrifuged and discard of the supernatant, the bacterial cells were washed once by 50%,100% acetone, twice in ethyl ether and air dried, the bacterial cell mass was weight and suspended in 0.1% formaline saline solution to a final concentration of 164 mg cells \ ml.

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

Group A constituted of 3 animals numbered as A1,A2,A3 each animal inoculated subcutaneously by 2 ml of vaccine 1 (Toxoid PLD) in the left of axillary region .The same dose was repeated after 4 weeks, group B constituted of 3 animals numbered as B1, B2, B3 each animal inoculated subcutaneously by 2 ml of vaccine 2 (Toxoid PLD +Bacterine) in the left of axillary region .The same dose was repeated after 4 weeks and group C constituted of 3 animals numbered as C1, C2, C3 each animal inoculated subcutaneously with 4ml saline adjuvant mixture.

Challenge exposure (Brogden, 1995)

One well identified colony of *C .pseudotuberculosis* isolates were inoculated in 250 ml brain heart infusion broth containing 0.1 Tween 80 and incubated at 37C° for 48 hours. 9 ml of brain heart broth with 1 ml culture broth and then serial dilution 1\10,1\100,1\1000 to measure optical density at 600 to find the concentration of 4 $\times 10^6$ forming unit per ml ,four weeks after second dose of vaccination ,each sheep was inoculated by s\c with 1 ml of diluted broth in the right axillary region.

Evaluation of vaccine efficiency

Post vaccinal reaction: Temperature was measured 3 days after inoculation of first dose, second dose and challenge of vaccine.

Antibodies assayed with ELISA: Blood samples were obtained from sheep before vaccination and at weekly intervals till the end of experiment (20 weeks). Antibodies were assayed in 96 ELISA plates as previously described by Ihab *et al*. (Ihab, 2016).

Scar evaluation of the developed regions: All animals were examined every 2 weeks to observe the appearance of any enlargements of external lymph nodes of sites of challenge inoculation ,all animals were euthanized and necropsied 12-14 week post last vaccination . Besides sites of challenge inoculation and internal organ ,lymph nodes were examined for internal abscesses , samples were collected from abscesses for reisolation of *C .pseudotuberculosis* .Examined L.N were divided into 5 groups and examined as described previously by Selim *et al*., (Selim, 2016), 4 external right, left prescapular, right, left prefemoral and fifth group contain all of internal organs (right & left popliteal ,bronchial, thoracic, inguinal, mesenteric). Abscess in inoculation site was considered as one unit. Each group is consider in case of scar evaluation, scar in each unit was calculated according to size, shape, appearance of lymph node.

RESULTS

Bacterial identification and Characterization

Out of 40 aspirates collected from sheep lymph node with caseous lymphadenitis like lesion ,20 isolated proved to be typical corynebacterium .Eight isolates exhibited the criteria *of C. pseudotuberculosis* colonies based on cultural, morphological and biochemical characters. All isolates are Gram-positive, non-sporulated, non motile pleomorphic, curved rods, catalase, urease positive and nitrate reduction negative.The bacteria ferment glucose, maltose and mannose but not ferment sucrose, lactose, or xylose. All the *C. pseudotuberculosis* isolated from sheep tested in this study showed synergistic hemolysis with *Rhodococcus equi* culture filtrate (Modified CAMP) that showed a clear zone of hemolysis surrounded the tested colonies.The exotoxin producer strain of *C. pseudotuberculosis* inhibited the hemolytic activity of staphylococci that appear as a wide clear zone of growth (Reverse CAMP Test).

Characterization of the isolates by Polymerase Chain Reaction

Products of the expected size (910 bp) were successfully obtained with DNA templates of 8 *C. pseudotuberculosis* isolates of sheep origin by using PCR as seen of figure 1 Characterization of phospholipase –D (PLD) by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot technique as seen in figure(2) was revealed that specific bands for PLD gene appear at 62 K.D ,the quantity of PLD protein in each strain was estimated using the gel pro software as seen in Table (1) .PLD =2.3 µg \10ml equivalent to 11.5 µg \ml.

Evaluation of the productive efficacy of vaccines formulations Clinical responses to vaccination: After challenge all sheep were lethargic for the first 72 hours then return to normal temperature *humeral immune response After 4 weeks from the first dose of vaccination, the second dose of vaccination and challenge.*

Table 1. Theanalysis of phospholipase D- PLD protein using SDS-polyacrylamide gel electrophoresis by pro gel software

Lane1			Lane3		Lane4		Lane5		Lane6		Lane7		Lane8		Lane9	
Raw	Mol.wei	Amount	Mol.wei	Amount	Mol.wei	Amount	Mol.wei	Amount	Mol.wei	Amount	Mol.wei	Amount	Mol.wei	Amount	Mol.wei	Amount
Raw1			198	1.8752	198	2.0185	198	2.3089	198	2.3195	198	2.8232	198	2.808	198	3.0741
Raw2	175	1.9484	155.83	1.4053	155.83	2.0185	155.83	2.4029	155.83	2.2338	155.83	2.3546	155.83	2.8728	155.83	2.3642
Raw3	83	2.1801														
Raw4	62	2.0153														
Raw5	47.5	2.4263														
Raw6	32.5	2.2439	31.838	4.2086	31.838	4.2292	31.838	3.9083	31.838	4.6561	31.838	4.0693	31.838	3.989	31.838	4.5722
Raw7	25	2.0624														
Raw8	16.5	1.6212														
Raw9			11.967	3.5945	12.156	3.0751	11.967	3.1184	11.967	3.2752	12.156	3.4693	11.967	3.4096	11.967	2.4501

Table 2. Mean OD and ± standard error of phospholipase –D antibody titer in sera of sheep before vaccination

Type of vaccine	Animal number	OD	Mean
Toxoid PLD (group A)	A1	0.161	0.163
	A2	0.167	
	A3	0.161	
Toxoid PLD+Bacterine (formalin killed bacteria)(Group B)	B1	0.172	0.154
	B2	0.115	
	B3	0.175	
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 sero negative.

Table 3. The mean value of phosoholipase –D- titer by using different types of vaccine

Type of vaccine	Period	N.	PLD antibody	
			Mean*	SE±
Control	FM	3	0.163	0.0080 b
Toxoid PLD	FM	3	0.707	0.1440a
Toxoid PLD +Bacterine	FM	3	0.611	0.0149a
Control	SM	3	0.162	0.0018b
Toxoid PLD	SM	3	0.763	0.1247a
Toxoid PLD +Bacterine	SM	3	0.658	0.0103a
Control	P.Chall	3	0.139	0.0035b
Toxoid PLD	P.Chall	3	0.690	0.0628 a
Toxoid PLD +Bacterine	P.Chall	3	0.624	0.0254a

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 differ

The mean optical density (OD) Values of antibody titer in sera of sheep vaccinated with vaccine 1 (group A) and vaccine 2 (groupe B) were higher than cut off value as seen in Table 2, Fig (3). Statistical analysis using GLM model as seen in Table 3 illustrate 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, second dose of vaccination, regardling the type of vaccine, the mean OD values of phospholipase –D antibody titer in sera of vaccinated sheep were not significance.

The post mortem findings of vaccinated sheep

The post mortem findings of sheep vaccinated with vaccine 1 (Toxoid PLD): This group included 3 sheep (A1,A2,A3) , post mortem finding of Animal number A1 no lesion of caseous lymphadenitis ,Animal number A2 showed that the right and left prescapular lymph node were enlarged and oedematous, Animal number A3 showed that the left prescapular lymph node was enlarged .The average of total score of lesions developed in all animals of this group was 5\54.

Table (4) Scores of lesions detected during post mortem examination of external and internal lymphnodes of sheep post challenge

Type of vaccine	Animal number	I.S	External L.N				Total score of internal L.N	Total score of each group	of % infection	of % infection
			RPS	lPS	RPF	LPF				
Toxoid PLD	A1	-0\3	-0\3	-0\3	-0\3	-0\3	-0\3	5\54	9	91
(group A)	A2	-0\3	++2\3	++2\3	-0\3	-0\3	-0\3			
	A3	-0\3	-0\3	-0\3	-0\3	-0\3	-0\3			
Toxoid	B1	-0\3	-0\3	+++3\3	-0\3	-0\3	-0\3	14\54	25	75
PLD+Bacterine	B2	-0\3	-0\3	++2\3	-0\3	++2\3	++2\3			
(group B)	B3	-0\3	++2\3	++2\3	-0\3	-0\3	-0\3			
Control	C1	+++3\3	+++3\3	+1\3	+++3\3	++2\3	+++3\3	40\54	74	26
unvaccinated	C2	+++3\3	+++3\3	++2\3	+++3\3	+1\3	++2\3			
((group C	C3	++2\3	+++3\3	+1\3	+++3\3	-0\3	++2\3			

external (lPS,RPS)right,leftprescapular ,(RPF,LPF)right ,left prefemoral

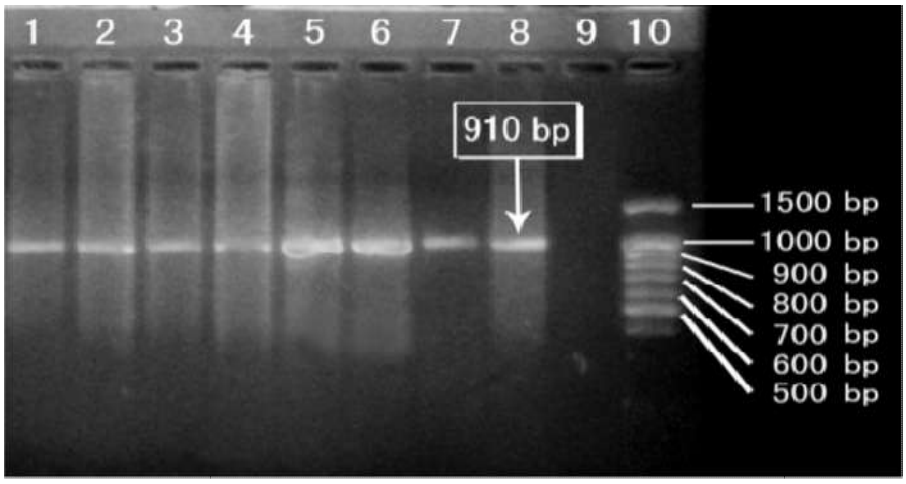

Figure 1. PCRof PLD amplification gene at 910 bp bands from 8 sheep isolates of *Pseudotuberculosis.* Lane 9 negative control, Lane 10 marker

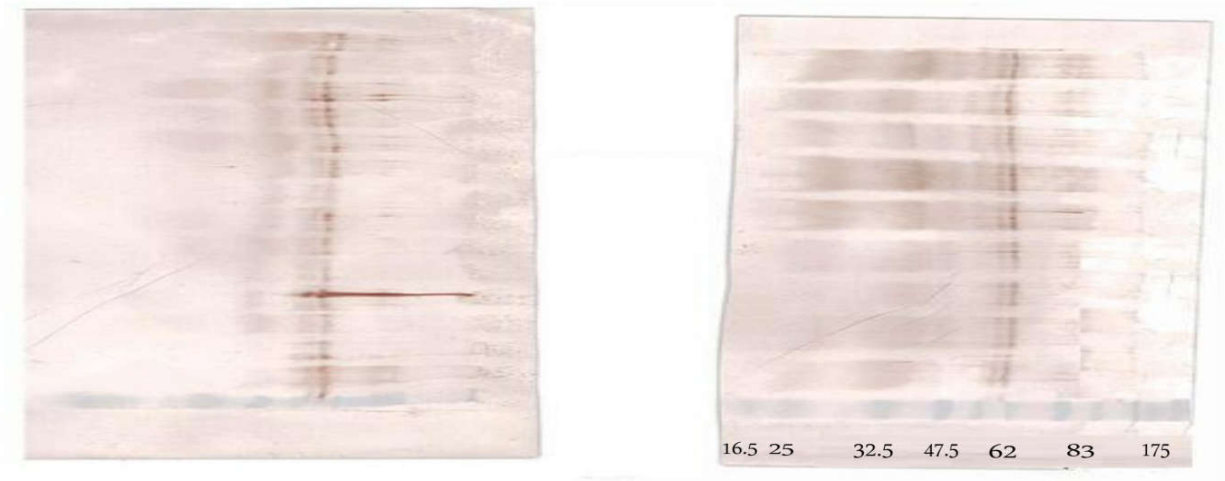

Figure 2. Imunoblot showing bands specific for PLD gene at 62 K.D from 8 sheep isolates no. (2,3,4,5,6,7,8,9) of *C.pseudotuberculosis*. Lane 1 positive control, Lane marker

This score revealead that the percent of infection 9% and protection was 91% as seen in Table 4.

The post mortem findings of sheep vaccinated with vaccine 2 Toxoid PLD plus bacterine

This group included 3 sheep (B1,B2,B3), post mortem finding of Animal number B1 showed that the left prescapular was enlarged and caseated, Animal number B2 showed that the left prescapular lymph node were enlarged and oedematous, the left prefemoral was enlarged and caseated, the left popliteal lymph node was enlarged and oedematous . Animal number B3 showed that the right and left prescapular lymph node was enlarged and oedmatous .The average of total score of lesions developed in all animals of this group was 14\54. This score revealed that the percent of infection 25% and protection was 75% as seen in Table 4.

Figure 3. That showed infected lymph node (prescapular) as deposition of calcium granules that form lamella in abscess

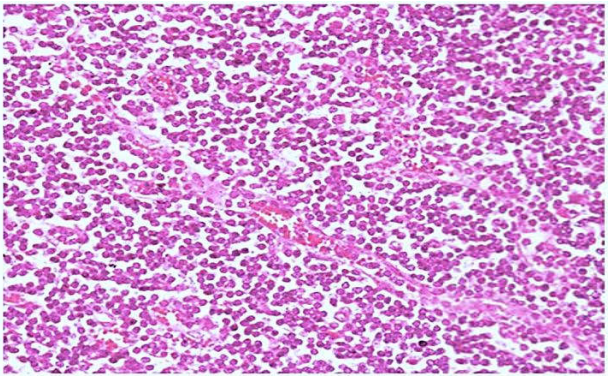

Figure 4. Histopathological examination of the lymph nodes

The post mortem findings of control unvaccinated sheep (group C): This group included 3 sheep (C1,C2,C3) All of which showed typical lesions of caseous Lymphadenitis as described previously in the last experiment by Selim *et al*,. (*Selim, 2016*). The average of total score of lesions developed in all animals of this group was 40\54 .This score revealead that the percent of infection 74% and protection was 26%.

Histopathological examination of the lymph nodes from sheep challenged with C pseudotuberculosis: The most prominent feature as seen in Figure 5 revealed multiple caseated granules (pseudotubercle granulane) with deposition of calcium salts in the entire necrotic tissue.

Bacteriological examination of infected lymph nodes and abscesses in inoculation site: *Corynebacterium pseudotuberculosis* was reisolated from the infected lympnodes.

DISCUSSION

Preparation of an efficient vaccine against caseous lymphadenitis (CLA) in sheep represents a challenge for researchers, because the protective antigens may be due to either exotoxine of the organism which called phospholipase – D(PLD) (Ellis, 1991), or due to immunogenic antigen present in the cell wall of this bacterium and the protoplasm of the microbial cells (Yozwiak, 1993 and Cameron, 1971). It is thought that the virulence *of C. pseudotuberculosis* enhanced by PLD that increasing vascular permeability so impairing neutrophil chemotaxis toward the site of infection to limiting bacterial opsonization, resulting in bacterial dissemination (Yozwiak, 1993). To prepare the vaccine against the local caseous lymphadenitis CLA, the causative organism *C. pseudotuberculosis* was isolated from aspirate of 40 diseased sheep showing abscessed lymph nodes suspected to be caseous lymphadenitis from Raas Seder Station and then 20 isolates were identified by cultural ,morphological and biochemical characterization .eight isolates showed the principle characteristics of *C. pseudotuberculosis* organism and confirmed the findings of (Barakat, 1984 and Brown, 1987).

The identification of isolates was confirmed by detection of PLD gene by PCR and all isolates were positive and showed amplification of specific bands at 910 bp molecular weight as shown in Figure (1) and agree with the data stated by (McNamara, 1994).All isolate were investigated for PLD activity by modified CAMP test .eight *C. pseudotuberculosis* isolates showed synergistic haemolysis together with *Rhodococcus equi* on blood agar .These findings confirmed the results of the previous investigations performed by exotoxin (Baird, 2007 and Selim, 2012), where they demonstrated that ceramide phosphate produced by the PLD converted to ceramide by the PLC produced by *Rhodococcus equi*,that cause disorganization the lipid bilayer and lysis of RBCs concerning their biological activities, all isolates were subjected to reverse CAMP test that showed large hemolytic zones around inhibit the hemolytic activity of *S. aureus* lysine these results agree with (Egen, 1989).

The SH (synergistic haemolytic) activity can be used as a predictive assay for the production of PLD by *C pseudotuberculosis*, but it is inadequate technique to detect the actual concentration of PLD in culture supernatants which can be achieved by SDS-PAGE and immunoblotting technique using highly specific anti-PLD antibodies and analyzed by the Gel pro computer software to maintain a proper amount of PLD antigen in each dose of toxoid vaccine as seen in Table (1), the PLD appear at molecular weight 62.5 KDa by Comassie blue staining as seen in Figure (2) and agree with Muckle *et al*., (Muckle, 1992). The first vaccine in the present study was toxoid PLD vaccine (vaccine A) prepared from highly virulent strain of *C pseudotuberculosis* ,the second vaccine formulation (vaccine 2) was prepared by combination of formaline –killed *C. pseudotuberculosis* cells with toxoid PLD. To increase the efficiency of the vaccine, addition of oil adjuvant composed of mineral oil and spain at a ratio of 9:1 respectively. Water in oil emulsion were known as the most effective adjuvants to generate high and durable antibody responses to vaccinal antigen following a single injection (Jansen, 2005). Water in oil emulsion retained the antigen at the site of injection, thereby performing a depot function by delaying the antigen absorption and preventing antigen release

that stimulate immune responses (Herbert, 1986). Span 80 is an ester widely used in food products and oral pharmaceuticals ; it is generally regarded as non-toxic and non-irritating additive and it is approved for use many countries (Brogden, 1996). The role of various adjuvants in improving immune response to *C pseudotuberculosis* vaccine was examined by many investigator that used as an adjuvant in a unique dose of 20 mg for induction an immune stimulant of the reticuloendothelial system. This adjuvant which called MDH was used in combination with the bacterial immunogenicity and potentates the immune response against intracellular bacterial infection (Woodard, 1980). In sheep, MDH has been used in low doses of 10 mg, increasing the efficacy of *C. pseudotuberculosis* cell wall vaccines (Brogden, 1990). All animals were lethargic for the first 72 after challenge then returned to normal temperature. This observation was recorded also by Piontkowski and Shivvers (Cameron, 1971). To evaluate the result of humeral immune response by ELISA in serum samples were collected from all animals at 4 times during the study :at zero time ,at 4 weeks after the first, second dose of vaccination and 4 weeks after the challenge with living *C. pseudotuberculosis* and the results was illustrated (in Tables (2,3), Figure 3,the results of antibody immune response of sheep vaccinated with vaccine (1)showed higher titer of PLD antibody .there was no significant increase in the group mean antibody titers following primary and secondary vaccination and after 4 weeks from challenge (Mean OD 0,706 ,0.763 and 0.690 respectively) Tables (3), Figure (3) which indicate the presence of anamnestic response due to previous sensitization of vaccinated animals with first and second doses of vaccination.

This result coincide with Selim, *et al*; Ellis *et al*., (Selim, 2010 and Ellis, 1991a), who reported that sheep vaccinated with toxoid PLD could stimulate the humeral immune response. The results of antibody immune response of sheep vaccinated with vaccine (2) revealed (Mean OD 0.611 ,0.658 and 0.624 respectively) by comparing these OD values with OD measured in animal vaccinated with PLD alone which were (0,706 ,0.763 and 0.690)as seen in Tables (3) ,Figure (3) respectively , it can be observed that the bacterial cells didn't improve the level of produced antibodies but to some extent decrease that level .This results agree with the conclusion of (Eggleton *et al*; Barga) (Eggleton, 1991 and Braga, 2007), where the last demonstrated that The cell-wall vaccinated alpacas showed a lesser degree of protection than toxoid vaccine with abscesses in internal and regional lymph nodes, but without symptoms ,these results disagree with Burrell; Piontkowski and Shvvers who reported that the addition of bacterial cells to toxoid vaccine may improve the protective efficacy of vaccination with toxoid vaccine alone (Burrell, 1980 and Piontkowski, 1998).

The post mortem findings of sheep vaccinated with vaccine toxoid PLD (vaccine 1) as seen in Table (4) revealed that the average total score of lesions developed in all animals was 5\54 with percent of infection 9%and protection 91%, this result concided with Eggleton *et al* (Eggleton, 1991) who recorded a 90% protection and Hodgson *et al* (Hodgson, 1999). who reported a 95 % protection. Talaa (Talaat, 2004), revealed a protection percent of 90%., while Selim *et al*., (Selim, 2010), detected only 80% of protection when used toxoid PLD vaccine. The post mortem findings of sheep vaccinated with vaccine 2 (formaline killed *C pseudotuberculosis* plus toxoid PLD as seen in Table (4) revealed that the average total score of lesions developed in all animals was 14\54 with percent of infection 25%and protection 75%.Killed vaccine is the least efficacious in preventing infection by facultative intracellular bacteria such as *C pseudotuberculosis* and generally induces only a humeral response .This results confirmed the findings of Pionthowski and Shivvers (Piontkowski, 1988) who evaluated the commercially available combined vaccine that contained inactivated whole cells and detoxified exotoxin and found that the 8 of 18 vaccinated sheep have external abscesses ,the same observation was also recorded by Eggleton *et al* (Eggleton, 1991a) who compared the protective potency of cell –free toxoid and toxoid formaline killed cells of *C pseudotuberculosis*, they found that the protective potency of the vaccines was not improved by the inoculation of cells of *C pseudotuberculosis*. On the other hand the post mortem finding of control unvaccinated sheep (group C) illustrated that the average total score of lesions that were shown in Figure (4) developed in all animals was 40\54 with percent of infection 74%and protection 26%.This results of experimental infection coincide with those of Eggleton *et al*. (Brogden, 1995), who reported that the percent of infection with living *C pseudotuberculosis* in unvaccinated group was 72%.On the other hand Eggleton *et al* (Eggleton, 1991a and Eggleton, 1991c), mentioned that the percent of infection in unvaccinated control group was 51% also Selim *et al*., (Selim, 2010) who reported that the percent of infection in unvaccinated control group was 80%,in contrast to Pintkowski and Shivers ; Hodgson *et al* (Piontkowski, 1988 and Hodgson, 1999), who revealed that the percent of infection with living unvaccinated control group was 100%. This variation in the susceptibility to infection with *C pseudotuberculosis* may be attributed to difference in the dose of the inoculated bacteria or to the individual variation of animals in relation to natural immunity.

Histopathological examination of the lymph nodes from sheep challenged with *C pseudotuberculosis* were performed in the present investigation as seen in Figure 5 .The most prominent feature revealed multiple caseated granules (pseudotubercle granulane) with deposition of calcium salts in the entire necrotic tissue, also concentric granules consisting of central caseation surrounded by a layer of lymphocytes and epithelial cells and encapsulated border of connective tissue. This was found in the control unvaccinated group D as described by Pepin *et al*; Ghanbrpour *et al*, they reported the dual role of granulomatous lesions in chronic bacterial infections although they limit bacterial dissemination ,the granules do not impair the persistence of infectious organism in the host, leading to focal tissue damage (Pépin, 1991 and Ganbarpour, 2002). Also, Mahmood *et al*. (Mahmood, 2015) observed severe abscess formation, congestion, hemorrhage, inflammatory cellular infiltration and degeneration and necrosis in Boer goats subcutaneously inoculated with *C. pseudotuberculosis* as compared to those inoculated intravenously with its phospholipase D. The results of this work showed that the most efficient vaccination against CLA was provided in animals vaccinated with toxoid PLD alone because PLD toxoid could stimulate humeral immune response resulting in high titer of anti PLD antibodies assayed by ELISA.

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