Vet.Med.J.,Giza. Vol.56, No.3 (2008) :239-253.

COMPARISON OF PROTECTION INDUCED BY *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* TOXOID AND BCG VACCINES IN LAMBS

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Received: 1. 12. 2008
Accepted: 5. 1. 2009

SUMMARY

A total of 460 sheep in 3 distinct age/sex groups were examined to determine the occurrence of caseous lymphadenitis in Beni Suef Governorate. The results confirmed that frequency increased with age but also revealed increases in extent of involvement and occurrence of visceral lesion, particularly in association with lesion in the body. An attempt was conducted to evaluate the immunogenic value of toxoid (prepared from *C. pseudotuberculosis* field isolate) and BCG used in sheep farms. Application of ELISA revealed slight increase in antibody response to toxoid and BCG vaccines prior to challenge, however, at week 7 (1 week post challenge) there was statistical significance elevation of antibody titre in group A vaccinated with toxoid over group B vaccinated with BCG ($P < 0.05$). The capacity for induction of memory is a better indicator of vaccine performance and is most effectively assessed by challenge in the natural host. The protection rates post challenge were 71.4 %, 42.9 % and 14.3 % for lambs immunized with toxoid , BCG and non vaccinated group respectively. Generally, the toxoid vaccine (prepared from field isolate) has been shown to confer high but not absolute degree of protection against caseous lymphadenitis and was more efficient than BCG. In sheep, it is likely that a short period of expression is insufficient to induce strong immune response in vivo.

INTRODUCTION

Caseous lymphadenitis is a chronic infectious disease of adult sheep and goat caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*), a Gram positive rod that produce phospholipase D exotoxin (Cyrillo et al., 2004 and McKean et al., 2007).

Two forms of the disease have been described; the external cutaneous form, typified by unilateral lymphadenopathy and the internal visceral form involving lymph nodes and/or organs within the body cavity (O'Reilly et al., 2008). Either form reduced milk production, weight gain and the infected sheep are culled from breeding flock due to unthriftiness or decrease fertility while their carcasses or part of carcasses are condemened at abattoir (Lea et al. , 1987 and Nowicki et al. , 2004).

Introduction of an infected animal into a non infected herd can result in a multiplicity of abscesses 2 or 3 years later. Once the disease is established, it is difficult to eradicate because drug therapy is not effective (Dorella et al., 2006).

Control programmes have traditionally involved detection lancing of abscesses and isolation of effected animals, disinfection of contaminated shears, docking equipment and dipping fluid and culling of animal with recurring abscesses. This method of control has not proven satisfactory due to long term survival of the bacteria in the environment shedding of bacteria in large numbers from ruptured abscesses and presence of undetectable internal abscesses which may be a source of infection (Menzies et al. , 1991 and Baird and Fontaline , 2007).

Vaccination of sheep and goats with *C. pseudotuberculosis* has given encouragement to the possibility of controlling caseous lymphadenitis (Brogden et al. 1984 and Rose et al. , 2002).

Exotoxin of *C. pseudotuberculosis* (a phospholipase D) breaks down sphinogomylin has a lytic effect in vitro on endothelial cells. The exotoxin may function in vivo as a permeability factor allowing for local-spread of the organism and increasing chances that the bacteria may be carried to more distant sites. The expression of phospholipase D by intercellular *C. pseudotuberculosis* was shown to play a small but a significant role in the reduction macrophage viability following infection and demonstrated that the regulation of *C. pseudotuberculosis* phospholipase D is complex . This regulatory complexity may play an important role in allowing the pathogen to successfully adopt to the challenging host-environment during infection, migration , establishment and disease progression. Antitoxin antibodies in the initial bacterial multiplication period may limit local spread and general dissimination of the organism (Brown et al. , 1986 and McKean et al. , 2007).

In Egypt, it is used to vaccinate sheep with Bacillus Calmette and Guerin (BCG) against *C. pseudotuberculosis.*

The purpose of the present study was to isolate and identify *C. pseudotuberculosis* from exudate of external abscesses and internal abscesses. The second objective was to determine the degree of protection induced in lambs by formalized *C.*

pseudotuberculosis exotoxin (toxoid of field isolate) and BCG vaccine by measuring the humoral immune response and vaccination challenge inoculation system.

MATERIALS AND METHODS

Animals :

A total of 460 sheep (132 lambs , 176 ewes and 152 rams) were examined to determine the occurrence of caseous lymphadenitis in Beni Suef Governorate.

Lambs:

An experimental study was conducted on 21 Osimi lambs between 10 to 12 weeks old to evaluate the efficiency of vaccines. Lambs were prescreened for the presence of antibodies to *C. pseudotuberculosis* by ELISA to ensure no prior caseous lymphadenitis exposure.

Isolation of *C. pseudotuberculosis* from sheep:

Diagnoses of external and internal abscesses were conducted by clinical appearance and postmortem examination. Swabs from organs with abscesses and lymph node were taken and transmitted with minimum of delay to the laboratory for bacteriological investigation. Swabs were streaked directly onto 5 % sheep blood agar and brain heart infusion agar. Plates were incubated for 24 -48 hrs. at 37°C. Isolates were identified to be *C. pseudotuberculosis* on the basis of colonial morphology, Gram stain and biochemical identification according to Carter and John (1990).

Preparation of toxoid (Brwon et al., 1986):

C. pseudotuberculosis isolate was cultivated on blood agar and incubated at 37°C for 48 hrs. Single *C. pseudotuberculosis* colony was removed from the blood agar plate, placed in 250 ml of brain heart infusion broth , incubated at 37°C with shaking for 72 hrs and allowed to settle at 4°C for 18 hrs. The broth culture was then centrifuged for 30 min. at 6000 rpm at 4°C. The supernatant was vacuum filtered through 0.45µm filter Merthiolate was added to the filtered supernatant to a final concentration of 1:10000. The resulting solution was stored at 4°C . The power and haemolytic activity of the prepared cultured culture filtrate was evaluated according to Attia (1994), culture filtrate was serially diluted (2 fold) and tested against sheep erythrocyte in 96 wells plate. The result expressed as the reciprocal of the dilution.

Quantitative measurements of synergistic haemolytic activity (Songer et al., 1988). Culture filtrate of *C. pseudotuberculosis* were tested with culture filtrate of *R. equi*, synergistic haemolysis was measured. This was done using 1.5 % agar in PBS containing 5% sheep erythrocyte and *R. equi* culture filtrate. Formaldehyde solution was added to small aliquots of toxin until a concentration of 3 % was attained.

Quality control analysis of the prepared vaccine was tested for inactivation, purity, sterility and safety according to the Standard International

Protocol described by Code of the American Fedral Regulation (1985).

BCG vaccine (freeze and dried) was supplied by Veterinary Serum and Vaccine Research Institute, Egypt.

Experimental design:

The lambs (21) were divided randomly into 3 groups (7 lambs / group). Group (A) was injected with 0.5 ml of toxoid and 0.5 ml of Freund's incomplete adjuvant subcutaneously, just caudal to scapula. Group (B) was vaccinated subcutaneously with 0.1 ml BCG and group (C) was the unvaccinated control group.

Blood samples were collected at the end of the 1st, 2nd and 3rd weeks post vaccination. The booster dose was given after 3rd week . Blood samples were also collected at the end of 1st, 2nd and 3rd week after boostering.

Challenge test :

Twenty one days after the last vaccination, the vaccinated and unvaccinated lambs were inoculated intradermally in the right paralumber fossa with 0.1 ml of a 48 hrs broth culture of *C. pseudotuberculosis* (3 X 10^6 CFU/ml). On the 6th and 12th weeks post challenge typical sites of colonization were examined and palpation of superficial lymph nodes. As intradermal inoculation sites suppurated, swabs of the exudates were collected and were cultured bacteriologically. Postmortem examination; lymph nodes and abdominal viscera were examined visually and by palpation. Enlarged lymph nodes or area suspected of having abscesses were incised and examined bacteriologically.

Measurement of humoral immune response was done by ELISA :

Detection of *C. pseudotuberculosis* specific antibodies in serum samples from the immunized lambs were measured by ELISA (Maki et al., 1985).

Antigen preparation :

A culture was grown in brain heart infusion broth to which 0.1 % Tween 80 was added. After 72 hrs incubation on a mechanical shaker, the culture was refrigerated at 4°C overnight. The supernatant broth was centrifuged and subsequently filtered through a membrane filter to remove all cells. This constituted the source of toxin which was preserved with 1:10000 merthiorate and kept at 4°C. 96 wells ELISA microtitre plates were coated with the prepared antigen (20µg/well) dissolved in carbonate bicarbonate buffer pH 9.6. The plates were then blocked with 5% skimmed milk. These plates were used for titration of the collected serum samples for *C.pseudotuberculosis* - specific antibodies. Serial two fold dilution from 1:2 to 1:256 of lamb sera were dispensed into the microtutre plates (50µl/well) and the plates were incubated at 37°C for 60min. The plates were washed 3 times using

PBS containing 0.05% Tween 20 (PBS-T). Horseradish peroxidase donkey antisheep IgG (BETHYL Laboratories, Inc.) diluted 1/1000 was dispensed in the wells (50µl)well). The plates were incubated at 37°C for 30 min and then washed 3 times using PBS-T. ABTS substrate (KPL, Gaithersburg MD20879, USA) was added (50µl/well) and incubated at 37°C for 15 min., then the reaction was stopped by addition of 25µl well 1% Sodium Dodocyl Sulphate (SDS). Plates were read using ELISA reader at 405nm. For detection of antibody titre in group B vaccinated with BCG, the ELISA plate was coated by BCG vaccine. Two control were used : conjugate added to antigen coated wells without serum as a control negative and control positive using anti *C. pseudotuberculosis* high titre serum. The cutoff point of positive and negative samples was of optical density 0.3 (Rose et al. , 2002).

Statistical analysis :

The data were statistically analyzed by Student's t-test with aid of the general linear model procedure of Statistical Analysis System (SAS, 1992).

RESULTS

Bacteriological examination of the pus swabs collected from external abscesses and internal organs revealed the recovery of *C. pseudotuberculosis* with an incidence of 15.2 %, 30.1 % and 28.3 % in lambs, ewes and rams respectively with an overall incidence of 25.2 % (Table 1 and Photos 1, 2 and 3).

The colonies were small, whitish, opaque surrounded by narrow zone of beta hemolysis on blood agar while the colonies were larger in size whitish more luxuriant on brain heart infusion agar. Biochemical identification revealed that the isolates were catalase and urease positive and nitrate negative.

The hemolytic activity of the culture filtrate was 128 units and the quantitive measurement of synergistic hemolysis was 8.5 mm.

Table (1) : Prevalence, sites of occurrence of lesion in caseous lymphadenitis affected sheep.

Sites of lesion	Lambs (132)		Ewes (176)		Rams (152)		Total (460)	
	No.	%*	No.	%*	No.	%*	No.	%**
Body only	17	12.9	34	19.3	26	17.1	77	16.7
Viscera only	3	2.27	7	4.0	8	5.3	18	3.9
Body + viscera	0	0	12	6.8	9	5.9	21	4.57
Total	20	15.2	53	30.1	43	28.3	116	25.2

* The percentage is calculated to the total number of each group of animals.
** The percentage is calculated to the total number of animals.

The quality control analysis of the prepared vaccine proved sterility, safety and the hemolytic activity was lost with 3 % formalin.

Antibody titre in the sera of lambs given either toxoid or BCG vaccine were monitored employing ELISA. The result as shown in Table (2) indicate that sera of group (A) lambs that received toxoid vaccine showed rise of antibody titre at the 1st and 2nd weeks after vaccination as the geometric mean antibody titre were 0.35 and 0.46 respectively. While, slight drop in antibody to 0.41 was observed at 3rd week post vaccination. After boostering, the antibody titres were increased to 0.50, 0.61 and 0.70 at 4th, 5th and 6th week post vaccination respectively. A similar patterns was observed in lambs given BCG vaccine . The geometric means of antibody titre were 0.35, 0.44, 0.40, 0.49, 0.62 and 0.70 in first six weeks respectively. Statistical analysis revealed that there was no significant difference between antibody titres in sera collected from lambs in group (A) vaccinated with toxoid and group (B) vaccinated with BCG vaccine.

It was noticed that the antibody response to toxoid and BCG vaccine was slight increased prior to challenge. However, at the 7th week (1st week post challenge) there was a statistical significance elevation in the antibody titre in group A vaccinated with toxoid over the group B vaccinated with BCG (Table 3). It is clear that toxoid is more efficient at inducing an anamenestic response as indicated by a significance elevated humoral response in the 4 weeks post challenge. Antibody responses at week 7 served as a good indicator of immunological memory which was characterized by accelerated and augmented antibody titre could clearly be distinguished from primary immune responses to *C. pseudotuberculosis* in the non vaccinated control group (Fig. 1).

All lambs had a febrile episode 24 to 48 hrs after challenge. During the next 6 weeks all lambs developed suppurative ulcerating lesions at the inoculation sites and enlarged prefemoral lymph node although the lambs remained clinically healthy. Protection was assessed at necropsy after 12 weeks of inoculation and protective status was conferred only if sterile immunity was achieved (total absence of lesion in different lymph nodes). Group (A) vaccinated with toxoid, 5 lambs did not have lesion and 2 lambs had abscesses in the right prefemoral lymph node one of them had abscesses in the lung while Group (B) vaccinated with BCG, 3 lambs did not have lesion, 2 lambs had abscesses in the right prefemoral lymph node and 2 lambs had multiple abscesses at the draining lymph node and at inoculation sites. Internal abscesses were found in lung and liver of one lamb. The protection rates were 71.4 % and 42.9 % for toxoid and BCG vaccines respectively (Fig. 2).

Group (C) unvaccinated and challenged, the protection rate was 14.3 %. An external lesions were found in the inoculation sites and were confined

the prefemoral and prescapular lymph nodes (6 abs). Few lesion were found in the internal lymph nodes (1) where as many lesion were found in the visceral organs especially in the lung (3) and liver (1).

Table (2) : Serum antibody optical density by ELISA among lambs vaccinated with *C. pseudotuberculosis* toxoid and BCG vaccines

	Weeks post vaccination					
	1	2	3	4	5	6
Toxoid	0.35	0.46	0.41	0.48	0.60	0.66
	0.42	0.49	0.49	0.57	0.68	0.70
	0.28	0.42	0.40	0.49	0.55	0.72
	0.44	0.57	0.46	0.53	0.64	0.75
	0.39	0.43	0.38	0.47	0.51	0.62
	0.37	0.49	0.45	0.52	0.63	0.69
	0.26	0.37	0.33	0.44	0.66	0.74
Geometric mean	0.35	0.46	0.41	0.50	0.61	0.70
St. Error	0.026	0.024	0.020	0.016	0.023	0.017
BCG	0.29	0.38	0.34	0.42	0.57	0.71
	0.31	0.39	0.32	0.46	0.62	0.78
	0.41	0.47	0.43	0.52	0.68	0.73
	0.37	0.42	0.41	0.50	0.69	0.72
	0.26	0.40	0.33	0.49	0.53	0.61
	0.39	0.45	0.44	0.48	0.59	0.64
	0.45	0.59	0.56	0.59	0.67	0.70
Geometric mean	0.35	0.44	0.40	0.49	0.62	0.70
St. Error	0.026	0.027	0.032	0.020	0.023	0.022
t-test-p (toxoid & BCG)	0.9295	0.7020	0.7923	0.8622	0.6566	0.9404
Control	0.15	0.07	0.04	0.07	0.02	0.09
	0.09	0.03	0.07	0.012	0.09	0.04
	0.07	0.12	0.09	0.011	0.03	0.11
	0.13	0.18	0.11	0.03	0.012	0.15
	0.02	0.05	0.13	0.02	0.018	0.06
	0.06	0.02	0.03	0.09	0.07	0.03
	0.08	0.06	0.02	0.07	0.04	0.02
Geometric mean	0.07	0.06	0.06	0.03	0.03	0.06
St. Error	0.02	0.02	0.02	0.01	0.01	0.02
t-test-p (Toxoid & control)	0.00007	0.000004	0.000006	0.000001	0.0000001	0.0000001
t-test-p (BCG & control)	0.000124	0.000042	0.000221	0.000001	0.000001	0.0000002

Data represented as geometric mean ± SE.
Significant at $P < 0.05$.

Table (3) : Serum antibody optical density of lambs vaccinated with toxoid, BCG and unvaccinated group after challenged by *C. pseudotuberculosis.*

	Weeks post challenge					
	1	2	3	4	5	6
Toxoid	5.26	5.32	3.12	2.81	1.87	0.92
	5.81	5.10	4.72	2.65	1.95	0.83
	4.75	4.47	3.98	3.20	1.14	1.04
	6.13	5.86	4.21	3.87	2.26	0.98
	5.79	4.30	3.18	2.11	1.65	0.92
	6.35	5.21	3.65	2.83	1.32	0.89
	5.52	4.11	3.22	2.94	1.85	0.72
Geometric mean	5.64	4.88	3.68	2.87	1.68	0.89
St. Error	0.204	0.239	0.230	0.203	0.145	0.039
BCG	3.13	2.95	2.10	1.96	1.53	0.71
	3.92	2.20	2.07	1.74	1.31	0.52
	3.16	2.06	1.85	1.32	1.22	0.49
	4.28	3.74	3.12	2.16	1.64	0.88
	4.56	3.28	3.08	2.53	1.27	0.47
	3.74	2.15	2.12	1.48	1.09	0.63
	4.85	3.27	2.94	1.82	1.55	0.56
Geometric mean	3.90	2.74	2.42	1.82	1.36	0.59
St. Error	0.20	0.20	0.17	0.12	0.06	0.04
t-test-p (toxoid & BCG)	0.0004	0.0007	0.0115	0.0102	0.0095	0.0030
Control	1.73	1.72	1.95	1.98	1.15	0.31
	0.94	1.24	1.44	1.76	1.31	0.27
	1.30	1.85	1.97	1.95	0.62	0.49
	1.14	1.27	1.73	1.87	0.70	0.36
	0.97	1.36	1.82	1.92	1.13	0.52
	0.85	1.91	1.40	1.22	0.93	0.46
	1.26	1.82	1.78	1.94	1.52	0.42
Geometric mean	1.14	1.57	1.71	1.79	1.01	0.39
St. Error	0.11	0.11	0.09	0.10	0.12	0.04
t-test-p (Toxoid & control)	0.0000	0.0000	0.0004	0.0024	0.0053	0.0000
t-test-p (BCG & control)	0.0001	0.0084	0.0141	0.7327	0.0484	0.0386

Data represented as geometric mean ± SE.
Significant at $P < 0.05$.

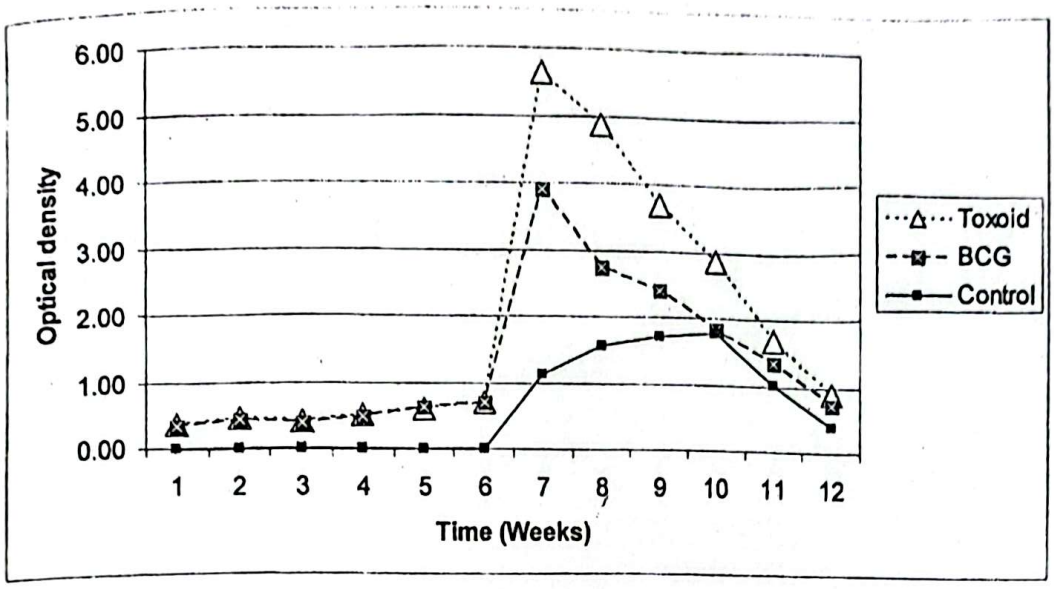

Fig. (1) : Humoral response in sheep vaccinated with toxoid, BCG and control group. All animals were challenged at week 6 with live *C. pseudotuberculosis*.

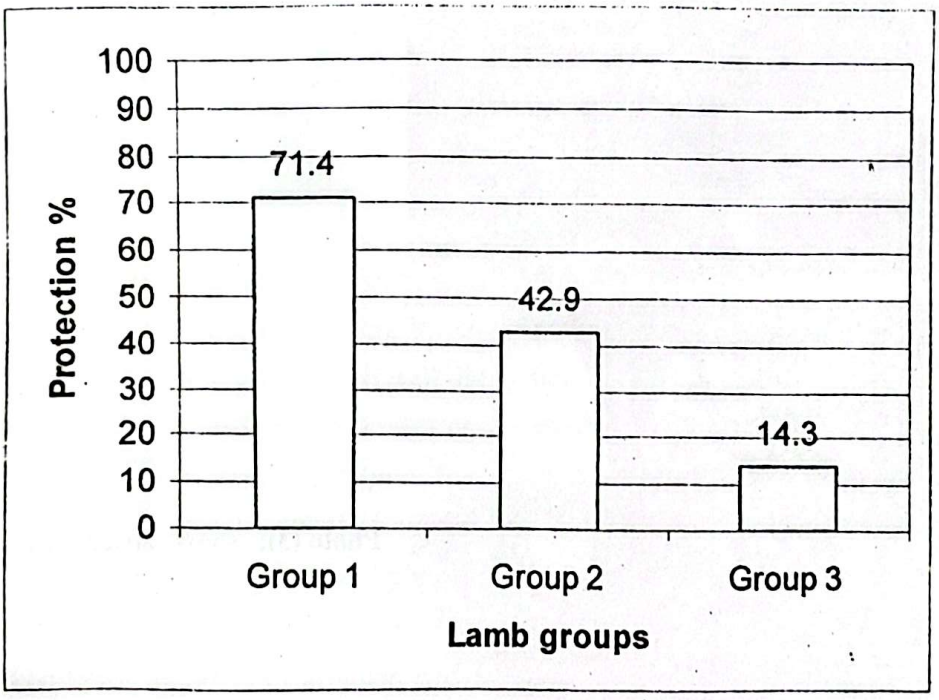

Fig. (2): Protection rate in lambs vaccinated with toxoid, BCG and unvaccinated group after challenged with homologous virulent strain of *C.pseudotuberculosis*.
Group(1): Immunized with toxoid.
Group (2): Immunized with BCG.
Group (3): Unvaccinated group.

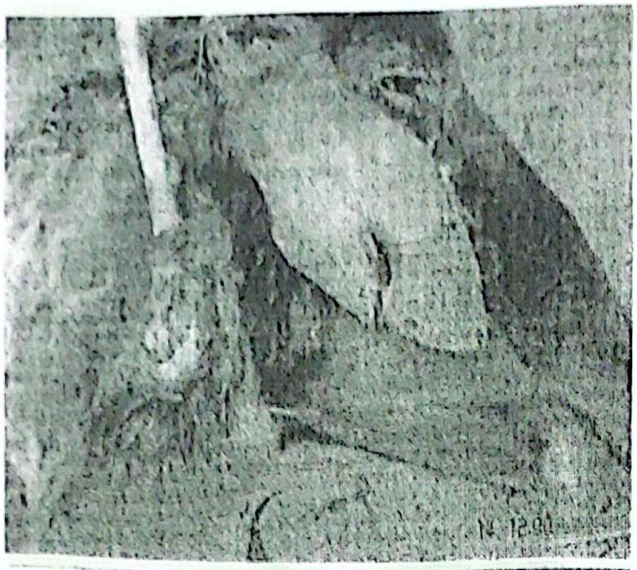

Photo (1): Shows external large abscess in the skin.

Photo (2): Shows abscess in the viscera. The contents of the abscess appeared caseated and yellowish in colour.

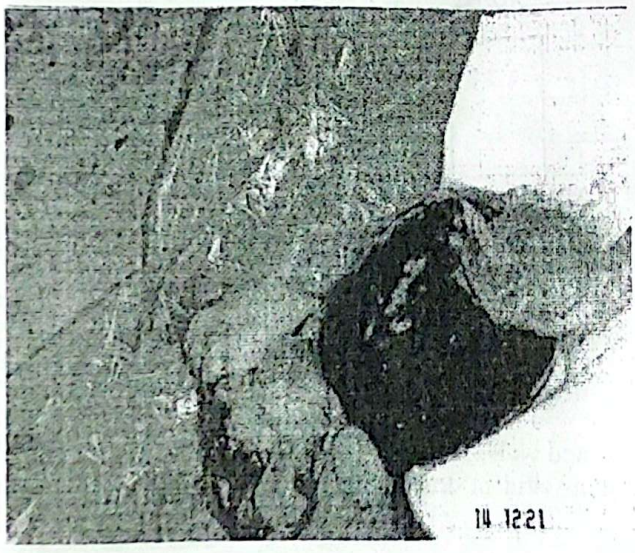

Photo (3): Shows abscess in the liver.

DISCUSSION

Caseous lymphadenitis has been a significant disease in majority of sheep rearing region for over a century (Baird and Fontaline, 2007). It is considered to have potential to develop into a major disease and financial problem in sheep industries. In this study, *C. pseudotuberculosis* was isolated from lambs, ewes and rams with an incidence of 15.2 %, 30.1 % and 28.3 % respectively (Table 1). The results confirmed that frequency increased with age and also revealed an increase in the extent of involvement and occurrence of visceral lesion, particularly in association with lesion in body (Photo 1, 2 and 3). These results coincide with Batey (1986). The role played by *C. pseudotuberculosis* in causing caseous lymphadenitis among sheep in Egypt is well known and reported by many authors (Mostafa and Afifi , 1996; Abd El-Gahani et al. , 1998 and Maarouf and Farag , 2008).

Control of caseous lymphadenitis by vaccination remains controversial (Papin et al., 1993). Vaccination of lambs with whole cell and cell wall did not stimulate immunity sufficient to protect entirely the lambs from infection with *C. pseudotuberculosis* (Brogdgen et al., 1984). However, substantial protection induced by toxoid preparation has been attributed to neutralization of permeability-increasing effect of exotoxin which subsequently reduce the spread of bacteria from the local site of primary infection (Papin et al., 1993). Use of vaccine containing a toxoid as the active constituent is practiced in many countries. The culture filtrate used for preparation of vaccine had a high hemolytic activity (128 units) and synergistic activity (8.5 mm). Isolates with a higher hemolytic activity were more virulent and pathogenic than week hemolytic strain (Mohammed et al. , 2001).

Serum samples from immunized lambs were monitored before and after challenge by ELISA. Although synergistic hemolysis inhibition test was specific in measuring anti-toxin to *C. pseudotuberculosis*, the ELISA procedure was easier, more convenient to operate and more sensitive (Maki et al., 1985).

Data represented in Table (2) illustrated that sera collected from lambs immunized with toxoid of *C. pseudotuberculosis* showed a non significant increase in antibody titre as compared to sera from lambs immunized with BCG vaccine during different intervals post immunization. In both groups of lambs antibody production started from day 7 post vaccination and marginally declined on day 21. Then slight rise in geometric means of antibody titre in the 4^{th}, 5^{th} and 6^{th} weeks post boostering. While vaccination can induce both effector and memory responses only effector responses are usually measured, however for most vaccines the capacity to generate memory is more important than their ability to induce effector responses. Indeed, the potential to rapidly respond

to a pathogen with strong and rapid response in the early stages of an infection will, in many cases confer protection. The capacity for induction of memory is therefore often a better indicator of vaccine performance and is most effectively assessed by challenge in the natural host (Rose et al., 2002). Immunological memory is characterized by accelerated recall response that is greater in magnitude compared to a primary response. These features of memory were particularly evident in the early postchallenge period at week 7 (1 week postchallenge). At this time point systemic antibody response to *C. pseudotuberculosis* infection in unvaccinated control lambs were low. However, in vaccinated lambs antibody titre was a higher and there was significant difference in antibody response between group A vaccinated with toxoid and group B vaccinated with BCG (Table 3 and Fig. 1). Ellis et al. (1991) reported that the toxoid used in various trails which is a *C. pseudotuberculosis* cell culture filtrate with documented exotoxin activity induce antibody responses to numerous bacterial antigens and Muckle et al. (1992) found in addition to the phospholipase D, 2 apparently cell associated immunodominant proteins of 68 and 12 kDa were detected in sera of infected animals . Fig. (1) showed rapid decline of antibody titre after the 7th week till 12 weeks in lambs vaccinated with toxoid indicated that *C. pseudotuberculosis* r eacted positively with specific antibody produced from vaccination. However, BCG provided a non specific immunity.

In the present study, there was a significant differences between toxoid vaccinated group and control group. Despite the high antibody titre all over the 6 weeks, in lambs vaccinated with BCG, there was no significance difference in 4th week postchallenge . This might be due to systemic antibody response to *C. pseudotuberculosis* infection in the non vaccinated lambs which increased.

Following challenge with virulent *C. pseudotuberculosis* vaccinated lambs developed fewer and less severe lesion than did unvaccinated lambs. The protection rate were 71.4 and 42.9 % for toxoid and BCG respectively (Fig. 2) and 14.3 % in unvaccinated group. These results are in agreement with Brown et al. (1986) who stated that, the antibodies to exotoxin during the first weeks after inoculation may protect against dissimination of *C. pseudotuberculosis.*

Generally, toxoid vaccine (prepared from field isolate) has been shown to confer a high but not absolute degree of protection against caseous lymphadenitis. It is more efficient than BCG. In sheep, it is likely that a short period of expression is insufficient to induce strong immune response in vivo. Further studies on cloning of the phospholipase D gene of *C. pseudotuberculosis* will be useful for clarifying the effective role of exotoxin in natural and experimentally induced infection.

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