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A Trial for Vaccination of Sheep against Caseous Lymphadentitis Using Oil Adjuvent Bacterin Enhanced by Bacillus Calmette–Guerin Vaccine

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ABSTRACT

Corynebacterium pseudotuberculosis is the etiologic agent of Caseous lymphadenitis, A disease that affects goats and sheep and can cause severe economic loss, Characterized by abscess development in subcutaneous tissues, Lymph nodes and internal organs.In this study we prepare a formalized vaccine from local strain combined with montanide oil adjuvant. The prepared vaccine was assessed in two groups of local balady bread sheep in comparison to a negative control group (5 animals each). Group I vaccinated with vaccine+BCG and group II vaccinated with the vaccine only. Both were in the same age, same sex and free from CLA lesions. Both had a booster dose at 21 day post vaccination. The samples were collected at days 0, 10, 21, 44, and 60 post vaccination. The cellular immune response was evaluated by phagocytic activity and index, and differential leukocytes count and the humeral immune response was evaluated by ELISA (Enzyme-linked immunosorbent assay) test. The result reveal a significant increase ($p<0.05$) in cellular and humeral immunity respond and prepared vaccine with BCG (Bacillus Calmette–Guerin vaccine) could be used for prevention and control of CLA (Caseous lymphadenitis) in sheep.

1. INTRODUCTION.

Corynebacterium pseudotuberculosis is a Gram positive facultative intracellular organism causing caseous lymphadenitis (CLA) in sheep and goats. The disease characterized by chronic suppurative inflammation and significant economic losses to the sheep industries worldwide (Paton et al., 2005; Dorella., 2006) The disease is oftenbecomes apparent during meat inspection (Lioyd, S. 1994), and it is manifested in two different forms: external CLA (abscesses in superficial lymph nodes and subcutaneous tissue) and visceral CLA (abscesses in internal organs)(Alonso et al.,1992; Paton et al.,1994; Stoops et al., 1984) .The disease causes significant economic losses due to decreased meat production, damaged wool and leather, as well as decreased reproductive efficacy. Once the disease is established, it is difficult to be treated because antibiotic therapy is not effective. So, massive vaccination may be necessary to reduce the diseases prevalence .many types of vaccines are present, Bacterin vaccines, Toxoid vaccines, combined vaccines, live vaccines and DNA vaccines. Killed C.p adjuvant vaccine has been used for Prevention and control of this disease, but its efficacy is variable and the reports about protection rates mostly variable. It was suggested that efficacy of killed adjuvanted vaccines depends mainly on the used adjuvant which is safe for use and able to enhance the vaccine Immunogenicity (Brogden et al., 1996; Cameron et al., 1973; Cameron et al., 1998). C.pseudotuberculosis well known as a facultative intracellular microorganism, so cell mediated immune response play a major role in protection and providing adequate protection against CLA (Fontaine et al.,2006). BCG could be helpful when used to improve the levels of immune response of sheep (Ebeid et al., 2011). The present study was directed to develop oil adjuvant vaccine from killed local isolated C.pseudotuberculosis field strain and evaluation of its humeral and cellular immunity response in sheep of the prepared vaccine only and combined with BCG.

2. MATERIALS AND METHODS:

2.1. Bacteria:

A local isolates of C. pseudotuberculosis was used to prepare the vaccine. It was obtained from unopened abscess in parotid lymph node of heavy infected balady ram from grooming herd at Matrouh Governorate.

2.1.1. Sample collection and Bacteriological examination and Identification of the isolates: After the incubation period ended, when bacterial multiplication occurred in the medium, staining by Gram stain was performed, and the bacterial species were identified by motility, morphology, fermentation of glucose, sucrose and lactose (TSI - triple sugar iron test), catalase, urease and nitrite reduction test according to (Quinn et al., 2005; Dorella et al., 2006; Koneman et al., 1992)

2.2. Vaccine preparation:

The vaccine prepared from locally isolated strain of c. pseudotuberculosis inactivated by formalin .killing or inactivation of C. pseudotuberculosis using formalin was carried out as the description of (Brown et al., 1986) for 2 days.

2.2.1.1Corynbacterium pseudotuberculosis bacterin: Bacteria were cultured (grown) in nutrient broth or brain heart infusion broth at 37 C^o with aeration to stationary phase (determined by optical density). The cells were inactivated with formalin (0.4%, vol/vol), centrifuged (at 7000 rpm for 20 min at 4 C^o) and resuspended in PBS (pH 7.2.Bacterial concentrations were determined by McFarland's Nephelometer standards tubes, adjusted to10^{10} bacteria/ml with sterile PBS, and stored at 4 C^o.

2.2.1.2. Sterility test of bacterin: Loop full of bacterin cultured on blood agar medium and No growth of bacteria occurred.

2.2.2. Formulation of vaccine*:* Bacterin and Montanide ISA 206 mineral oil adjuvant: **(**Barnett et al., 2002), Emulsification of equal volume of bacterin with equal volume of montanide ISA 206 mineral oil adjuvant by magnetic stirrer device (250 rpm/min for 15 minutes).according to (Iyer et al., 2001). Dose of sheep in this vaccine is 2ml.

2.2.3. BCG vaccine: BCG vaccine supplied by Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt. Used for vaccination of sheep intradermally using sheep dose of (0.1ml).

2.3. Animals:

Fifteen native sheep of about 6-8 months of age were apparently healthy with no history of caseous lymphadenitis kept under clinical observation for one month. All sheep were selected for the experiment had negative ELISA test. Sheep were divided into three groups (Five animals/ group) as follow:

Group I: Vaccinated with 0.1ml of BCG injected intradermally, simultaneously with the first dose of 2ml of the prepare vaccine injected S/C, then booster dose injected 21 days apart.

Group II: Vaccinated with 2ml of prepared vaccine then booster dose injected 21 days apart.

Group III: Control group.

2.4. Postvaccinal reaction:

2.4.1. Temperature: body temperature was obtained three days after vaccination.

2.4.2. Abscessation: clinical observation of the animals was carried out through the study to notes any abscess at site of injection.

2.5. Sample collection:

2.5.1. Blood samples: were collected in plastic test tubes containing heparin (100 I.U/ml) as anticoagulant and tested directly.

2.5.2. Serum samples: 5ml blood sample were collected from jugular vein at at days 0, 10, 21, 44, and 60 post vaccination from each sheep of the groups. And then centrifuged at 5000r/m centrifuge to obtain the serum. Then serum was separated in clean Appendorff tubes by using plastic pipette, finally serum samples were kept at -20 C^o until tested

2.6. Evaluation of protection efficiency of the prepared vaccine formula:

2.6.1. Determination of the cellular immune response:

2.6.1.1Phagocytic activity and phagocytic index : The Phagocytic activity and index was measured according to the method described by(Lehrer and Clins ,1989; Kawahara et al., 1991)

2.6.1.1.1 Reagents for phagocytosis measuring:

a. *Candida albicans.* Dept. Of Poultry and Fish diseases, Fac. Vet. Med, Alex. University, Egypt.

b. Crystal violet stain.

2.6.1.1.2. The amount of 50 µg candida albicans culture was added to one ml of blood collected from animals and shaken in water bath at 23-25 C^o for 3-5 hours. Smears of the blood were stained with Giemsa solution. Phagocytosis was estimated by determining the proportion of macrophages which contained intracellular yeast cells in a random count of 300 macrophages and expressed as percentage of Phagocytic activity (PA). The number of phagocytized organisms was counted in the phagocytic cells and called phagocytic index (PI). Results were expressed as means ± S.E.

Phagocytic activity (PA) = percentage of phagocytic cells containing yeast cells.

Phagocytic index (PI) =

Number of yeast cells phagocytized/ Number of phagocytic cells

2.6.1.2 Differential leukocytic count: Blood film was prepared according to described method by (Lucky, 1977). Diluted Giemsa's stain solution was poured over the film and left for 20 minutes, then rinsed in water current and examined by oil immersion lens. The percentage and absolute value for each type of cells were calculated according to (Schalm, 1986).

2.6.2. Determination of humeral immune response using ELISA:

ELISA was carried out according to its assay procedures prescribed on its catalog .we use (Qualitative Sheep Corynebacterium Pseudotuberculosis Antibody (Anti-CP) ELISA Kit, produced by Mybiosource laps. The absorbance values at 450 nm obtained using ELISA reader.

2.8. Statistical analysis: The obtained data were statistically analyzed for means and significance between the groups using ANOVA according to (Snedecor and Cochran, 1982) by using SPSS computerized system.

3. RESULT:

3.1Bacterial identification and Characterization:

The isolates was Gram-positive, non-sporulated, non-motile pleomorphic, curved rods, catalase, urease positive and nitrate reduction negative. The bacteria ferment glucose, but not ferment sucrose and lactose.3.2 clinical finding:

3.2. Postvaccinal reaction:

Body temperature: there was slight increase in body temperature (39.5°-40°) in vaccinated animals for two days after first dose then return to normal, no increase reported after the booster, only two animal develop sterile swelling at the vaccination site in vaccinated groups

3.3 Cellular immune response:

3.3.1. Phagocytic activity and Phagocytic index:

The result showed at Table (1), there was a significant difference (P<0.05) between groupI (vaccine + BCG) (2.36±0.301) and groupII (vaccine only) (1.86±0.11) and groupIII (control) (1.5±0.14).

Table 1: Cell mediated immune response of vaccinated sheep with C. Pseudotuberculosis vaccine as measured by phagocytic activity and phagocytic index.

Group	day	phagocytic activity±SE	Phagocytic index± SE
GroupI	Zero day	55%	1.6
	Day 10	66%	2.8
	Day 21	71%	2.9
	Day 44	69%	2.2
	Mean	65.25 % (±3.5) [b]	2.36± (0.301) [b]
GroupII	Zero day	56%	1.7
	Day 10	65%	2.2
	Day 21	61%	1.8
	Day 44	62%	1.8
	Mean	61% (±1.8) [ab]	1.86± (0.11) [ab]
GroupIII	Zero day	56%	1.5
	Day 10	56%	1.7
	Day 21	55%	1.5
	Day 44	55%	1.5
	Mean	55.5 %(±0.28) [a]	1.5± (0.14) [a]

The superscript alphabetical a, b indicate significant difference between group1 and control group (P<0.05) by using Duncan test.

3.3.2 Differential leukocytes count:

Differential leukocytes count result is showed at Table (2).there was a markedly increase in the lymphocytes accounts at day 10 in groupII (vaccine+BCG), return to normal at day 21 sample.

3.4. Humeral Immune response:

All sheep were negative on ELISA and no antibody titer was recorded at zero day (table 3) The mean optical density (OD) Values of antibody titer in sera of sheep vaccinated with vaccine +BCG (group I) and vaccine only (groupII) were higher than cut off value, Statistical analysis using ANOVA model illustrate that there was a highly significant (p<0.05) difference among the mean OD values of antibody titer in sera of vaccinated and control.

Table2: Cell mediated immune response as measured by differential leukocytic count Group Day Lymphocytes Monocytes Basophils Eosinophils Neutrophils

Group	Day	Lymphocytes	Monocytes	Basophils	Eosinophils	Neutrophils
GI	Zero day	48-49	2	7-8	6-7-8	34-35-36
	Day 10	55	1	4	3	37
	Day 21	46	1	8	6	39
	Day 44	48-49	2	7-8	6-7-8	34-35-3
GII	Zero day	47	2	7-8	6-7	4-35
	Day 10	54	1	5	6	34
	Day 21	47	2	7	7	37
	Day 44	47-48-49	2	7-8	6-7-8	35-36
GIII	Zero day	48	2	7-8	6-7-8	35-36
	Day 10	48	2	7	7	34
	Day 21	47	1	7	6	35
	Day 44	48	2	7-8	6-7-8	35-36

G I: GroupI (vaccine+BCG), G II: GroupII (vaccine only), G II: GroupIII (control).

Table 3: Humeral Immune response by Mean OD ± SE through the study

Group	zero day	21day	44day	60day
GroupI	0. 136^{a} (±0.0061)	0.397^{c} (±0.029)	0.394^{b} (±0.0033)	0.371^{c}(±0.0074)
GroupII	0. 149^{a}(±0.0055)	0.372^{b} (±0.0027)	0.382^{b}(±0.0051)	0.332^{b}(±0.014)
Group III	0.143^{a}(±0.0079)	0.133^{a} (±0.0032)	0.145^{a}(±0.0041)	0.145^{a}(±0.0069)

OD: optical density of colored reaction, cut of value =0.215 .The superscript alphabetical a, b and c indicate significant difference between vaccinated groups and control group (P<0.05) by using Duncan test

4. 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. The search of a formulation for production of an ideal vaccine against CLA, with less production costs and less side effect was attracted many C. pseudotuberculosis researchers for induction of long acting and strong vaccine (Paton et al., 2005; Dorella, 2006). In this study, we combined between killed bacteria vaccine with oil adjuvant, with BCG. In this study we used field virulent strain obtained from heavily infected sheep, same as (Ebeid et al., 2011; Ihab et al., 2016; Sohier, 2017; Ghazy, 2017). ,(Garg,1985) used attenuated strain 137E (received from Dr. C. H. Gallagher, Dept. of Veterinary Pathology, University of Sydney, New South Wales, Australia) was derived from a virulent strain (137C) by repeated in vitro passage on solid medium. (Brogden, 1996) used C.Pseudotuberculosis ATCC 194 10. (Fontaine, 2006) used UK virulent field (Braga, 2007) used a preserved C. pseudotuberculosis sample from an early passage of strain H0676.(İzgür,2010)taken a forward step, as he isolated their local virulent strains and then classified it according to its high hemolytic titer (HT) of exotoxins, then he choosed the most virulent local strain to use in vaccine preparation.

Killing or inactivating of bacteria in our study was by formalin (4%) same as (Fontaine, 2006; İzgür, 2010; Ihab et al., 2016; Sohier, 2017). (Garg, 1985) sonicated the cells for 30 min in an ice bath with standard probe of (Braun sonic 1510) at 250-300 watts. (Braga, 2007) used pulse sonication at 60% output for 10 min (Fisher-Sonic cell Disrupter). (Moura-Costa et al., 2008) sonicated the cells at 60 Hz, using five cycles of 60 s each (Branson Sonifier 450). (Ebeid et al., 2011) prepared four vaccines; two of them was prepared by formalin the others by Binary ethyleneimine solution. (Ghazy, 2017) used Binary ethyleneimine solution. The use of oil

adjuvants (Montanide ISA 206) resulted in enhancement of the immune response as it have favorable characteristics of low viscosity, lower reactivity and high potency (Abdelwanis et al., 2001; Barnett et al., 2002) stated that Montanide ISA 206 adjuvant induce an earlier and higher immune response.In our study we used (Montanide ISA 206) as adjuvant, same as (Ghazy, 2017), (Brogden, 1996) used light mineral oil as adjuvant (0.10 ml per dose). (Fontaine, 2006) used physiological saline as adjuvant that was because he want to insure that there was no effect of the adjuvant on the host resistance to infection. (Braga, 2007) used (MDP-Sigma–Aldrich) as adjuvant. (Moura-Costa et al., 2008) used Freund's incomplete adjuvant (Sigma–Aldrich). (İzgür, 2010) used Freund's incomplete adjuvant (Sigma–Aldrich). (Ebeid et al., 2011) prepared four vaccines, two of them was prepared by (Montanide ISA 206), the others by paraffin oil adjuvant. (Ihab et al., 2016) used oil adjuvant without determination. (Sohier,2017) used Water in oil emulsion adjuvant for preparation of vaccine which 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%.

C.pseudotuberculosis well known as a facultative intracellular microorganism, so cell mediated immune play a major role in protection and providing adequate protection against CLA (Fontaine et al., 2006). For inducing high cellular immune, we use BCG as non-specific Cellular immunostimulant, same as (Ebeid et al., 2011;Barakat et al., 1979;Osman et al., 2016) and going far as they used only BCG in a trails to evaluate its potency against CLA.As in our study we combined between vaccine and BCG, (Selim et al., 2016) combined between CLA vaccine of his preparation and Clostridial Vaccines, the same component of the GlanvacTM, was released in 1983 (Eggleton et al., 1991a).The clinical observations reveal slight increase in temperature for two days for all vaccinated animals, all animals returned to their normal activities within 3-4 days post vaccination. This result supported by those of (Ghani et al., 2016) who reported moderate increase in rectal temperature (40.24 ± 0.45°C) after vaccination for 3 days. Slight localized swelling was noticed at the sight injection in only two animals and disappeared within one week. This result Supported by those of (Ghani et al., 2016) who reported the same finding and to (Brogden et al., 1996) who revealed that vaccines containing 10 mg of the whole cell induced sterile abscesses detectable at the vaccination site in vaccinated lambs but disappear when the concentration of whole cells lowered to one mg, and to (Ebeid et al., 2011) who reported that transient mild swelling was developed at the vaccination sites which subsided within 7-14 days except one animal in group-1 and two in group-3which showed moderate abscessation at the site of vaccine injection, and for a short period after the 2nd vaccination, there was depression, febrile reaction and three of ten vaccinated animals showed transient fibrous swellings at the site of vaccination ,our result also confirmed the findings of (Piontkowski and Shivvers, 1998), who evaluated the commercially available combined vaccine that contained inactivated whole cells, detoxified exotoxin, and found that the eight of 18 vaccinated sheep have external abscesses. However, in contrast to (Ghazy et al., 2017) who reported that there was no abscessation at vaccination injection site and to (Eman, 2007) who mentioned that vaccinated animals remained clinically healthy after the first vaccination.For evaluation of cellular immunity respond, we use phagocytosis activity, index, and leucocytes different count. (Garg, 1985) used Specific migration inhibition of peripheral leukocytes to indicate cellular immune response of the sheep. (Ebeid et al., 2011) used lymphocyte proliferation assay, it was applied according to (Lee, 1984). (Ghani et al., 2016) evaluated cellular immune respond by Delayed Type of hypersensitivity (DTH) skin test.(Ghazy et al., 2017) used lymphocyte proliferation assay, it was applied according to (Lee, 1984). The cellular respond result revealed a significant increase in phagocytosis in groupI vaccinated with vaccine+BCG (2.36±0.301) in compare with groupII (vaccine only) (1.86±0.11) and control group (1.5±0.14), as showed in table 1. There was a markedly increase in the lymphocytes counts at day 10 in groupI (vaccine+BCG) reach(55),return to normal at day 21 sample , which present in Table (2). Increase in lymphocytes counts beside the increase in phagocytosis is the signs of induced cellular immunity, this results agree with this reported by (Ebeid et al., 2011) who declared that the

optical density of cell mediated immune response of vaccinated sheep with the different prepared C. pseudotuberculosis vaccine increased in the second week of vaccination and reached its peak by the 1st week post the second dose (3rd week post the first vaccination) indicating that BCG followed by 50µg toxoid with 20 mg formalized bacterin adjuvanted with Mantonid oil induced the highest optical density values of cell mediated immune response in vaccinated sheep and (Osman et al., 2016) who reported that the BCG vaccine induced cellular immunity plus increase in body weight of vaccinated offspring. In regards to using BCG as non-specific Cellular immunostimulant heterogeneous Vaccine, (Cameron and Fatthj, 1984) reported that immunization with BCG alone had no protective effect against caseous lymphadenitis, in contrast with (Barakat et al., 1979) who concluded that BCG can be used alone for vaccination against caseous lymphadenitis where it induced protection of 90% in lambs under natural condition of infection.There was a significant difference between the cellular immune response stimulated by the prepared vaccine. And significant differences were found between the vaccinated groups and no vaccinated one confirming that the prepared vaccine are capable to induce cellular immune response which play a role in protection against the organism as it is facultative intracellular pathogen as reported by (Cameron et al.,1998;Youssef ,2004). To evaluate humeral immunity respond, we used Qualitative Sheep Corynebacterium Pseudotuberculosis Antibody (Anti-CP) ELISA Kit, produced by Mybiosource laps,At zero day, the ELISA results was (0.136), (0.149) and (0.143) for groupI, II and III respectively. These results are less than cut off value (0.215) that mean all animals was serongative at the beginning of the study and free from CLA infection. The ELISA results reveal significant increase in antibody titer at vaccinated group (I and II) in compare with control group (III).mean OD reach (0.257) and (0.259) and (0.140) for group I and II and II respectively at day 10 . Reach its peak at 21 day post vaccine with (0.397), (0.372) for groupI and II, in contrast to (0.133) for control group ($p>0.05$), and remain high with slight decrease at day 60 with (0.371) (0.332) for group I and II respectively. (Cut off value=0.215).This result is slight less than this appear with (Ghazy et al., 2017) who use bacterin inactivated by binary ethylamine with montanid ISA206 as adjuvant with (1.045±0.08) at 4 week post vaccine. But resemble results that announce by (Ebeid et al., 2011) with (0.365) at 22 day post second dose by using Toxoid + Bacterium inactivated by BEI + Paraffin oil, (Ghani et al., 2016) who use commercial CLA vaccine with mean OD (1.984 ± 0.776) at 8th week post vaccination and (Osman et al., 2016) who use BCG only with mean OD (0.548) 2mounth post vaccination. It is also slight less than this appear with (Ihab et al., 2016) with (0.670) (cut off value 0.400), (Sohier, 2017) with peak (0.658) (cut off value 0.312) using Toxoid PLD +Bacterin, result was after second dose of vaccination in second month. In comparing with other trails who depend on killing whole cell in producing their vaccines, we use different strains, different procedures for inactivation, different types of adjuvants, different ways to evaluate the cellular immunity respond even different ELISA Kits from different laboratories with different cut off values, and even many of them create his own ELISA. This is the reason behind varieties in results of humeral immunity evaluation, but even this variation, the protection levels of all vaccines seem to be Convergent.

Trials preformed to produce CLA vaccine using PLD toxins depending on the fact that PLD is the most virulent factor of C.psuedotuperclosi reveal mighty success in constrict the disease and preventing dissemination of the bacteria from site of inoculation (Piontkowski and Shivvers, 1998).but expression and purification of PLD need a very expensive and advanced molecular biological techniques, (Fontaine et al., 2006). This is very expensive and hardly to preformed according to possibilities of sheep producer and breeder society in Egypt, although, in the same study, (Fontaine et al., 2006) report same protection levels by using simple formalin killed bacterin. In addition, it is reported that more side effects appear by using PLD vaccine as abcessiation in site of injection and Possibility of inducing signs of infection. (Eman, 2007). According to conviction that cellular immunity play a role in protection against the organism as it is facultative intracellular pathogen as reported by (Cameron et al., 1998;Youssef, 2004) and our results of high and durable cellular immunity respond,

beside our results of high titre of antibody against C.P cell wall antigen, we concluded that BCG plus formalin killed bacterin adjuvanted with montanide ISA 206 is likely to use as appropriate vaccine with simple preparation procedures and obtainable materials , same as only licensed vaccine strategy in UK , Currently in the UK, the only vaccination strategy permissioned is for the use of so-called autogenous" C. pseudotuberculosis bacterin vaccines. Autogenous vaccines are flock-specific, in that they are prepared using isolates from defined flock outbreaks, and are permitted for use only in the single holding from which they originated.

Such variation between the control group results and vaccinated groups, which was significantly different, confirming that the prepared vaccine antigens assessed a significant level of protection against infection. These results come in agreement and Supported by those of (Brogden et al., 1996; Hodgson et al., 1999)

5. CONCLUSION:

It is concluded that the prepared inactivated C. pseudotuberculosis vaccine adjuvanted with montanide oil ISA 206 associated with BCG vaccine proved to induce cellular and humeral immunity and could be used for prevention and control of CLA in sheep.

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