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Bacteriological, molecular and histopathological evaluation of four vaccines’ protective role against caseous lymphadenitis in sheep

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Abstract---The caseous lymphadenitis disease (CLA) due to Corynebacterium pseudotuberculosis has worldwide distribution and indicates high prevalence in different countries. The bacterium has many pathogenicity factors; firstly, “phospholipase D”, an exotoxin virulence factor that enhances vascular permeability and facilitates bacterial transmission causing endothelial cell damage. The bacterium's exterior lipid cover is the second pathogenicity factor. This protects it from hydrolytic enzymes found in the phagocytes of the host, where the bacteria proliferate and then release after rupturing. Bacterial proliferation causes CLA abscesses, which are followed by attraction and the formation of an inflammatory response, which increases lymph flow and vascular permeability. The purpose of this study was to look at postmortem findings, bacterial cultures, and histology of vaccinated sheep to see how well they were protected. On
15 male local sheep bred, four prepared vaccinations against *C. pseudotuberculosis* biotype 1 were tested. The animals were divided into 5 groups; four of various vaccines used and the fifth were kept non-vaccinated. A live virulent strain of *C. pseudotuberculosis* was given to each group. Unvaccinated animals displayed CLA symptoms similar to those seen in spontaneously sick animals. Postmortem examination findings, standard bacteriological culture methods were used to isolate *C. pseudotuberculosis*, as well as tissue preparation for histopathological sections. Abscess development in the superficial and hepatic lymph nodes was the most common results. Histopathological sections showed apparently normal lymph nodes of vaccinated sheep groups while non-vaccinated lambs had numerous caseated granules with calcium salt deposition of an onion-like appearance. *C. pseudotuberculosis* was reisolated from infected lesions of lymph nodes and visceral organ of both vaccinated and unvaccinated sheep and identified by synergistic hemolysis assay “CAMP” and PCR.

**Keywords**—*Corynebacterium pseudotuberculosis*, phospholipase D, molecular, histopathological, caseous lymphadenitis disease.

**Introduction**

The Gram-positive bacterium *Corynebacterium pseudotuberculosis* causes caseous lymphadenitis (CL), enzootic pseudotuberculosis is a chronic bacterial infectious illness that affects sheep and goats. *C. pseudotuberculosis* is the cause of numerous economic losses in sheep and goat flocks and herds (Williamson 2001). The *C. pseudotuberculosis* usually disseminated in sheep and goats and results in significant economic losses to farmers due to reduced milk yield, less meat production, loss of fertility, increased culling and condemnation of affected animals (Williamson 2001; Guimaraes et al. 2011). *C. pseudotuberculosis* often produces multi-virulence factors, the first important one is “phospholipase D”, an exotoxin virulence factor that enhances vascular permeability and facilitates bacterial transmission causing endothelial cell damage. The bacterium’s exterior lipid cover is the second pathogenicity factor. This protects it from hydrolytic enzymes found in the phagocytes of the host, where the bacteria proliferate and then release after rupturing (Flores-Díaz et al. 2016).

In a histology analysis of sick goats’ lymph nodes that were obviously changed, with *C. pseudotuberculosis*. Brown et al. (1986) reported the presence of an extensive central mass of eosinophils and dead macrophages on the superficial lymph nodes, linked with bacterial dispersion, surrounded by a layer of fibrous tissue containing clusters of lymphocytes. Coccobacilli with gram-positive staining can be found throughout the core necrotic mass, which is assumed to be a typical granulomatous lesion. Walker et al. observed intact MHC class II cells, debris in the core necrotic mass, and lymphocytes inside encapsulated lesions in a study of *C. pseudotuberculosis* pyogranulomas (Walker et al. 1991). They discovered a high abundance of CD8, as well as CD4 and CD5+ (pan-T) cells, in the lymphoid tissue of CLA lesions. This is thought to be a part of an antibacterial
defense system that prevents infected macrophages from congregating (Modlin et al. 1988).

Indeed, Guilloteau et al. (1990) concluded that the evolution of experimental the infection of *C. pseudotuberculosis* in superficial granulomas in sheep can be separated into two phases: the early phase, in which phagocytic cells migrate to the infection site, which can sometimes eliminate the pathogen; and the second phase, Due to bacterial persistence, macrophages and lymphocytes congregate at the inflammatory site, resulting in a localized lesion. Hard (1969) investigated whether activated peritoneal macrophages can increase *C. pseudotuberculosis* ability to destroy it while also allowing it to tolerate its cytotoxic effects. Alongside the cell’s borders, engulfed bacteria were discovered near groupings of primary lysosomes (Tashjian and Campbell 1983).

CLA produces swelling in the supramammary, submandibular, prescapular, popliteal, and prefemoral lymph nodes, as well as visceral organs such as the lungs (Binns et al. 2007; Radostits et al. 2007; Fontaine and Baird 2008). In animals, post mortem indicators Green yellow exudates from infected superficial lymph nodes were included (Radostits et al. 2007). The pus in the lymph nodes appeared smooth in the early stages, but it eventually turned lamellate (onion ring or onion skin), which is a pathognomonic sign of CLA (Radostits et al. 2007; Fontaine and Baird 2008). To detect CLA, histopathological examination of lymph nodes and/or conventional bacterial culture from intact lymph nodes are employed (Radostits et al. 2007; Smith 2002). In our investigation we studied necropsy examination findings, bacterial cultures and histology for sheep injected with four distinct vaccine formulations to elicit protection against caseous lymphadenitis, as we previously evaluated the humeral and cell mediated immune response to the four vaccines (Selim et al. 2016; Syame et al. 2017).

**Material and Methods**

**Vaccine formulations**

All vaccines were previously prepared and investigated by our previous study (Selim et al. 2016; Syame et al. 2017) as follow

**Animals**

To rule out the positive reactors, ELISA was used to test fifteen sheep aged 8–10 months. Negative ELISA (CLA-free) animals were separated into five groups, four of which were vaccinated with four vaccines, a fifth group of non-vaccinated pigs was exposed to the deadly biovar 1 strain.

**Vaccine 1 (group A)**

*C. pseudotuberculosis* toxoid culture filtrate Preparation of culture filtrate from isolated strain according to (Brown et al. 1986) with changes as indicated in previous research (Selim et al. 2016; Syame et al. 2017) Each dose (2ml) included 23mg PLD after being blended with 1 ml oil adjuvant.
**Vaccine 2 (group B) with formalin, Toxoid PLD vaccine (Toxoid + Bacterine) eliminated C. pseudotuberculosis:** (Syame et al. 2017)

Brogden et al. (1990) modified in their preparation of bacterin (formalized killed). *C. pseudotuberculosis* biovar 1 was used to make it (sheep origin). Each dose (2ml of bacterin) contained 164mg of cells and ml of bacteria that had been destroyed. To generate a 2mL bacterin dose, the combination vaccination was mixed with 1mL of oil adjuvant.

**Vaccine 3 (group C)**

**Toxoid PLD with clostridial Vaccine (Covaccine 8)** (Eggleton et al. 1991; Sohier 2006; Selim et al. 2016). The PLD filtrate was formalin inactivated, and 64g of it contained 23g PLD. The method was repeated using 40ml of covaccine 8 (Schering Plough Animal Health); an imported vaccine manufactured from a mixture of clostridial toxins and 6g of lyophilized powders of generated PLD culture, as described by Selim et al (2016).

**Vaccine 4 (group D)**

**Combination of Toxoid PLD with Clostridial Vaccine (Polyvalent Clostridial Vaccine):** Mixture of forty mL polyvalent clostridial vaccine prepared by Veterinary Serum and Vaccine Research Institute, Abbasia and 6g lyophilized powder of prepared PLD culture filtrate, as described by Selim et al (2016).

**Vaccination and Experimental Challenge**

The first four sheep groups from 1 to 4 were vaccinated with combination vaccines 1, 2, 3, and 4 respectively, 2ml dose subcutaneously be inoculated in the neck at middle region. The inoculated animals were re-immunized again with the same vaccines three weeks later. The vaccinated five group animals and the non-vaccinated control sheep were challenged three weeks after the last vaccination with virulent biovar 1 sheep strain in 2ml suspension containing 4X 106 CFU given intradermal as 1ml on both sides of the neck.

**Bacteriological examination**

Samples were taken from each lesion using cotton swabs in closed lesions or aspiration in open lesions. All samples were obtained in an aseptic environment and prepared as direct smear and causative agent re-isolation.

**Histopathological studies on the affected lymph nodes from challenged sheep** (Pepin et al. 1991)

**Tissues processing**

During the first 24 hours after sample collection, lymph nodes from the prescapular, prefemoral, and popliteal lymph nodes were dehydrated in stepwise ethanol (70-100 percent), cleansed with xylol, infiltrated, and implanted. These samples were placed in sterile, screw-capped bottles containing a 10% neutral buffered formalin solution and transported to the lab for histological evaluation. Drury and Wallington (1980) recommended that five micron thick paraffin slices
be made and stained with hematoxylin and eosin before being inspected microscopically.

**Re-isolation and identification of C. pseudotuberculosis by CAMP Test**

Infected lesions from vaccinated and unvaccinated sheep lymph nodes and visceral organs were collected and cultured in brain heart infusion agar plate supplemented with 5% sheep erythrocytes. A loopful of *R. equi* culture (kindly supplied by Prof. Dr. S. A. Selim Professor of Microbiology) was vertically streaked on the same plate, then incubated for 48 hours. *C. pseudotuberculosis* isolates were found to have synergistic hemolysis with *R. equi* (CAMP Test) (Songer et al. 1990).

**Laboratory diagnostic methods**

In this investigation, DNA was extracted from the lesion using molecular PCR Protocol B of the G-spin kit (iNtRON, South Korea). As indicated by the manufacturer, A 1.5ml Eppendorf tube was filled with 25mg of homogenized tissue sample, 200ml of Buffer CL, 20ml proteinase K, and 5ml RNase. 200ml of Buffer BL was added after 15 minutes at 56°C, mixed, incubated for 5 minutes at 70°C, and then centrifuged for 5 minutes at 13,000 rpm with the up-lysed tissue particles removed. A 400µl supernatant was mixed gently and transferred to a new Eppendorf tube with 200µl absolute ethanol. The material was fed to the spin column after 1 minute of centrifugation at 13,000 rpm. 700µl Buffer WA was added to the spin column, centrifuged for 1 minute at 13,000 rpm, then centrifuged for another minute at 13,000 rpm to remove the infiltrate. The spin column was then filled with a 50µl Buffer CE solution and transferred to a new numbered Eppendorf tube, where it was incubated at room temperature for 1 minute before centrifuging at 13,000 rpm for 1 minute. Finally, the sample infiltration was stored by freezing it at -40°C.

**The following primers and PCR profiles were used to molecularly identify C. pseudotuberculosis isolates**

Using the oligonucleotide primers used in this study, the PLD genes of C. pseudotuberculosis were discovered. PLD R5': CGC AAG CTT TCA CCA and PLD F5': CGG CCC GGG ATT ATG GCG ATC ATG CTT C3' For C. pseudotuberculosis PLD genes, CGG GTT ATC CGC T 3' oligonucleotide primers may amplify 930 base pair fragments. According to Cetinkaya et al. (2002), the PCR reactions were carried out. For gene amplification, a total of 30 l PCR reaction mixture containing DNA template, appropriate primers, and 1X master mix was utilised. For amplification, We used the following PCR conditions: a 5-minute first denaturation cycle at 94°C, 30 cycles at 94°C for 35 seconds each, annealing at 55°C for 45 seconds, primers elongation at 72°C for 45 seconds, and a final extension cycle at 72°C for 5 minutes. On a 1% agarose gel stained with ethidium bromide, the amplified PCR products could be detected.
Results

Postmortem examination findings

Macroscopic Examination

The most prevalent finding was abscess formation in the superficial cervical (prescapular) lymph nodes, as well as subiliac and parotid ones (Fig. 1). Following the removal of the afflicted lymph nodes, a thick fibrous wall encircled caseated thick whitish purulent material. The same tumors were found in the hepatic lymph nodes, and uninfected sheep’s lungs were affected, as shown in (Fig. 2, 3). A fibrous capsule encased the caseous substance the lymph nodes, which was light green in color.

Fig 1. Caseous lymphadenitis lesions, external form

Fig 2. CLA abscesses of unvaccinated sheep challenge with \textit{Corynebacterium pseudotuberculosis} in parenchyma of the lung

Fig 3. CLA abscesses of unvaccinated sheep challenge with \textit{Corynebacterium pseudotuberculosis} in the liver, abscesses in variable size are randomly distributed throughout all lobes
The lymph nodes of sheep infected with *C. pseudotuberculosis* were examined histopathologically

At first (Fig. 4) showed lymph node of vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* showing apparently normal lymph node, while in (Fig. 5) showed concentrically lamellated pseudo-tubercle granules with central caseation, in (Fig. 6,7).

Fig 4. Lymph node of vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* showing apparently normal lymph node (arrows)

Fig 5. Lymph node of vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* showing concentrically lamellated pseudotubercle granules with central caseation and peripheral connective tissues capsule enclosed in lymphocyte and epithelial cell

Fig 6. Lymph node of non-vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* showing numerous caseated granules (pseudotubercle granules) with calcium salt accumulation throughout necrotic tissue
Lymph nodes of non-vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* were showing numerous caseated granules (pseudotubercle granules) with calcium salt accumulation throughout necrotic tissue, histological (transverse section) was seen in (Fig. 8) of Lymph node of non-vaccinated sheep challenge with *Corynebacterium pseudotuberculosis*. Onion-like typical appearance was shown in (Fig. 9) Lymph node of non-vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* have large suppurative granuloma with consecutive layers.
Fig 9. Lymph node of non vaccinated sheep challenge with *Corynebacterium pseudotuberculosis* showing large suppurative granuloma. The classic onion-like pattern is formed by successive layers

**Reisolation and identification of C. pseudotuberculosis by PCR and CAMP Test**

*Corynebacterium pseudotuberculosis* was reisolated from infected lesions of lymph node and visceral organ of vaccinated and unvaccinated sheep. The isolates of *Corynebacterium pseudotuberculosis* were synergistic hemolysis with *R. equi* strain in “CAMP” as seen in (Fig. 10).

Fig 10. Synergistic haemolysis between (1) Rhodococcus equi and (2) Corynebacterium pseudotuberculosis

**Polymerase Chain Reaction (PCR)**

PCR was used to successfully generate products of the desired size (910 bp) from DNA templates of 8 *C. pseudotuberculosis* isolates, as shown in (Fig. 11).
Discussion

Because the PLD toxoid could elicit cellular immune response which be assayed by the lymphocyte proliferation. It had the top stimulating (SI) index ‘9.12’ with high antibody titer, the most effective immunization against CLA was given to animals vaccinated with toxoid PLD alone (Selim et al. 2016; Syame et al. 2017; Syame et al. 2018). However, vaccination with a merged clostridial toxoid with PLD toxoid vaccine, ‘either imported or locally prepared’ may provide protection against *C. pseudotuberculosis* challenge, though to a lesser degree than sole vaccination of PLD; SI became lower ‘5.73’ in combined vaccine recipients. (Syame et al. 2018). At the same time, the decline in antitoxin levels in antibodies and the stimulation index reversed on sheep’s percent protection against *C. pseudotuberculosis* challenge, dropping from 90% to around 80%. (Selim et al. 2016; Syame et al. 2017). In the current study, histological analysis of afflicted lymph nodes in vaccinated and uninfected sheep revealed pyogranulomas surrounded by neutrophil fragments and significant macrophage infiltration (Kuria et al. 2001). as evidenced by (Fig. 1). Macrophages are the most prominent cells in the lymphatic zone and necrotic center, where the intracellular pathogen *C. pseudotuberculosis* is present, and thus serve as antigen-presenting cells and antibacterial effector cells that can generate antibodies that neutralize the toxin of *C. pseudotuberculosis* (Walker et al. 1991; Pepin et al. 1994b).

After infection, vascular permeability increases with continuous infiltration of inflammatory cells, where phagocytic cells engulf the organism because its outer lipid structure allows it to survive, carry it to a lymph node to produce exotoxin, and the recurrent continuous. The release of organisms occurs as a result of the process of bacterial proliferation and cell death to visceral organs (Fig. 2, 3) and lymph nodes (Baird and Fontaine 2007). The results obtained and the establishment of the characteristic CLA lymph node abscess (Fig. 5, 6, 7, 8, 9) are compatible with Khater et al. (1984), Zaituun and Bayoumi (1994). The pyogranulomas development can reduce bacterial dissemination at the same time stimulate effective immune reactions (Pepin et al. 1991). (Fig. 1) showed a cross section of the afflicted superficial lymph nodes, which displayed an exterior thick fibrous capsule surrounded with thick caseated greenish pus. (Fig. 8) histological...
(transverse slice) of lymph node of non-vaccinated sheep challenged with *C. pseudotuberculosis*, Al-Gaabary and El-Sheikh came up with similar findings (2002).

Baird and Fontaine both argue that (2007), the animal's body produces a fibrous capsule as a defence mechanism, and the organism produces green pigment, which gives the pus its green colour. The majority of internal lesions in unvaccinated sheep were observed in the lungs (Brogden et al. 1984; Fontaine et al. 2006) and the liver, hepatic lymph node (Fig. 3). Renshaw et al. (1979) were in agreement, however Batey et al. (1986), Brown et al. (1987), and Pointon et al. (1988) were not (2019). The most common visceral lesions were discovered in lung and thoracic lymph nodes, according to Malone et al. (2002) and Malone et al. (2006). Infected lymph nodes and abscesses in inoculation sites were reisolated with *C. pseudotuberculosis*. On blood agar, isolates of *C. pseudotuberculosis* demonstrated synergistic hemolysis with *R. equi*, as shown in (Fig. 10). These findings backed up previous studies by exotoxin, which found that the PLD produces ceramide phosphate, which is converted to ceramide by the PLC produced by *R. equi*, causing lipid bilayer rupture and RBC lysis in terms of biological activity (Williamson 2001 and Selim et al. 2012). Molecular identification of *C. pseudotuberculosis* isolates which were recovered from vaccinated and non-vaccinated sheep were achieved successfully, this result was coincided with that reported by Mancini et al. (2012) and Schlicher et al. (2021).

**Conclusion**

The present study clearly demonstrates that the four vaccines that used in Egypt for protection against CLA have a considerable protective role in vaccinated sheep displayed in postmortem and histopathological pictures even when the organism was reisolated among the both vaccinated and non-vaccinated sheep with PCR confirmation.

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**Conflict of interest**

The authors declared that no conflict of interest.

**Authors Contribution**

Syame, M. Syame designated the study and experimental steps, performed the molecular analysis. Hussein A. Abuelhag, Mai M. Kandil and Gaber, E. S. assisted in the experimental study and bacteriological examination. Eman A. Ebessy performed post mortem and histopathological examination.
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