Association between fatty acids profile of
*Corynebacterium pseudotuberculosis* with
pathogenicity in mice

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Dedication

To my Father, Mother and
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Abstract

This study was done to isolate \textit{corynebacterium pseudotuberculosis} the causative agent of caseous lymphadenitis in sheep and to determine the quality and quantity of short chain fatty acids among the isolates to study the possible association between fatty acids with pathogenicity in mice.

In Al-Huda slaughterhouse at Omdurman Province 3875 sheep carcasses were examined for the presence of abscesses. By manual palpation only 67 (1.37\%) head of sheep were found to have frank enlargement in the superficial lymph nodes. At post-mortem examination 129 (3.32\%) carcasses were found to have one to three abscesses each.

A total of 157 lesions were collected and examined bacteriologically. \textit{C. pseudotuberculosis} was isolated and identified to the species level from 49 sample. Anti-beta haemolysin inhibition test was done and all strains tested were positive. Qualitative and quantitative analysis for short chain fatty acids among 20 isolates was done by using Gas liquid chromatography. Propionic acid was found in all isolates and formed the highest peak, but Valaric acid was not found. The differences in percentage composition of fatty acids has considerable taxonomic value to differentiate the isolates. Molecular characterization for 5 isolates of \textit{C. pseudotuberculosis} by PCR was attempted using primer targeting the 16r RNA gene.

These five strains were used to study the pathogenicity in mice and we found that the most pathogenic strains had high concentration of Propionic acid while the non pathogenic strain has low concentration.

In view of that, we put forward, the fact that the content of this fatty acid might contribute with other factors in virulence.
anguard report

وزنها الفضي ينتمي إلى فئة تراكم السائل في الجلد. وقد أجريت دراسة تتناول هذا الموضوع. في حالة الكاذب السير، فإن السبب لأنتفاخ الجلد قد يكون بسبب إنتاج النمط النوعي والكمية والتيارات المبعزة في القدح. حيث أن اثنين من النظم النوعية العامة للبربري في المرضى (11.73% من النموذج 129). وتساهم النموذج 93% في مثل هذه النتائج. النموذج 157 يوزع على 49 نوعًا. وعندما يتم قسمة هذه النتائج على النموذج 20، يوجد نسبة عالية من النموذج 10 في النموذج 20، بينما يوجد نسبة عالية من النموذج 15 في النموذج 10. يمكن أن تكون النتائج هذه مفيدة للدراسات الفردية أو الخلايا في المرضى. حيث أن اثنين من النظم النوعية العامة للبربري في المرضى (11.73% من النموذج 129). وتساهم النموذج 93% في مثل هذه النتائج. النموذج 157 يوزع على 49 نوعًا. وعندما يتم قسمة هذه النتائج على النموذج 20، يوجد نسبة عالية من النموذج 10 في النموذج 20، بينما يوجد نسبة عالية من النموذج 15 في النموذج 10. يمكن أن تكون النتائج هذه مفيدة للدراسات الفردية أو الخلايا في المرضى.
Introduction

According to official estimation, Sudan in 1994-95 had 103 million heads of livestock, sheep, cattle, goats and camels comprise the major meat-producing animals. Live animals are continuously in demand from the Arab countries because of their healthy attractive appearance, clean skin, and conformity to international specifications. The vast natural pastures scattered all over the Sudan provide meat free of chemicals and hormones. However, this important trade has been threatened by the occurrence of caseous lymphadenitis (CLA) among exported sheep. Ships are continuously returned from ports of entry in importing countries due to the disease. Thus inflicting heavy financial losses on sheep exporters and national economy. In Sudan, in spite of massive economic losses, no control measures are available at present. Antibiotic treatment is ineffective because viable \textit{C. pseudotuberculosis} remains protected by the capsule bounding the abscess.

This had led recently to an excitement and an upsurge in the study of the aetiology of caseous lymphadenitis with the aim to control the disease by vaccination or strict control measures. The main objectives of the present study were as follows:-

1. To isolate \textit{C. pseudotuberculosis} and study the bacteriological characterization of the organism.
2. To determine the percentage composition of short fatty acid of the isolates by using gas liquid chromatography.
3. To confirm the identification of isolates by PCR.
4. To study the possible association between short chain fatty acids with pathogenicity in mice.
Chapter one
Review of Literature

1.1. History:

In 1888, the French bacteriologist Edward Nocard isolated an unusual organism from a case of lymphangitis in a cow (Nocard, 1896). Some 3 years later, the Bulgarian bacteriologist Hugo von Preïsz identified a similar bacterium in cultures from a renal abscess in a ewe (Preïsz and Guinard, 1891). As a consequence of these related discoveries, the organism in question became known as the “Preïsz–Nocard” bacillus, a vernacular name with which it was linked for decades thereafter.

Towards the end of the 19th century the bacterium was described by the German bacteriologists Lehmann and Neumann in the first edition of their bacteriological atlas (Lehmann and Neumann, 1896). In that publication, the Preïsz–Nocard bacillus was renamed Bacillus pseudotuberculosis—a derivation of the Greek pseudes tuberculosis or “false tuberculosis” and a reference to the supposed clinical similarity of the lesions to caseous nodules of mycobacterial tuberculosis. In the First Edition of Bergey's Manual of Determinative Bacteriology, published in 1923, the organism was placed in the Corynebacterium genus, which had originally been created as a category for the human pathogen Corynebacterium diphtheriae. This edition of the Manual referred to work showing that B. pseudotuberculosis resembled C. diphtheriae in
morphology and cell wall composition, leading to a further change of name to *Corynebacterium ovis*. Subsequently, however, the organism was isolated from purulent infections and ulcerative lymphangitis in other mammalian species, including goats, horses and human beings. In recognition of this, by the time the Sixth Edition of Bergey's *Manual* was published in 1948, the species name had been changed back from *ovis* to the earlier designation of *pseudotuberculosis*. Since that point, the officially recognized designation of *Corynebacterium pseudotuberculosis* has remained consistent, notwithstanding the fact that several authors continued to refer to the organism as *C. ovis* until the 1980s.

1.2. Geographical Distribution:

The global range of *C. pseudotuberculosis* reflects that of farmed small ruminants, the bacterium having been identified in Europe, Australasia, North and South America, Africa and the Middle East (Robins, 1991; Paton *et al.*, 2005). In many of these countries CLA has been an established and economically important infection of livestock, particularly sheep, for decades. As early as the 1930s it was acknowledged that the disease “very extensively affected” the flocks of most mutton-exporting countries (Cesari, 1930).

Some suggestions have been made that the origins of the infection may lie in Europe, and that spread of *C. pseudotuberculosis* around the world followed the exportation of sheep by the 18th-century colonial powers. The Merino breed, which originated in Spain, was widely valued as a dual meat and wool animal and was exported extensively, first to South Africa and subsequently to Australia and the Americas. It has been suggested that this early exportation may, at the very least, have assisted in the spread of *C. pseudotuberculosis* (Paton, 2000). Such a theory is
difficult to prove, but supporting evidence might be provided by demonstration of a close genotypic relationship between isolates from different parts of the world.

Research interest in CLA was renewed in the 1970s, when the authorities in the USA, Canada and Japan applied strict regulations relating to the presence of lesions in imported sheep carcases. A further series of studies into disease pathogenesis and epidemiology was then initiated in Australia, which, as a major exporter of sheep and a high CLA prevalence within its national flock, had much to lose from the stricter regulatory regime. This in turn led to the formulation of control strategies aimed at reducing disease prevalence and provided a catalyst for developments in the field of vaccination.

1.2.1. Caseous lymphadenitis in Sudan:

In Sudan the disease in sheep was first reported in the year 1914 from Sennar. Since that time the disease was associated with economic losses in a variety of ways which include meat condemnation and downgrading of hides. Labour cost and antibiotics used to combating the disease are additional factors of economical loss.

1.3. Bacterial Characteristics:

1.3.1. Classification:

The Corynebacteriaceae are now considered to belong to the Actinomycetaceae, a family that also contains the Mycobacterium, Rhodococcus and Nocardia genera (Clarridge and Spiegel, 1995). C. pseudotuberculosis possesses many of the classical features of its genus (Collins and Cummins, 1986). It consists of non-motile pleomorphic rods
(0.5–0.6 µm by 1.0–3.0 µm) that are Gram-positive, although such staining may sometimes be irregular. Groups of the bacteria tend to show a characteristic palisade or “Chinese letter” arrangement in smears.

At a temperature of 37 °C, *C. pseudotuberculosis* will grow under aerobic or anaerobic conditions. On solid media, the bacterial colonies are pale in colour, dry and friable in consistency, and may be moved freely over the surface of the agar with the point of a probe (Quinn *et al.*, 1994). After incubation for 24 h, small yellowish colonies will appear, increasing to a diameter of 1–2 mm after 48 h (Coyle *et al.*, 1985). Bacterial growth benefits from the addition of serum or whole blood to nutrient media. When whole blood is used, a narrow band of β-haemolysis is seen around each colony, although it may appear only after incubation for 48–72 h.

When grown in liquid media or when in aqueous suspension, *C. pseudotuberculosis* has a tendency to form clumps. This has been related to the presence of long-chain 2-branched 3-hydroxy fatty acids (so-called “mycolic acids”), on the outside of the cell wall (Carne *et al.*, 1956). Mycolic acids were first identified in 1939, in the tubercle bacillus (Asselineau and Lanéelle, 1998) and were subsequently found to be a feature common to the actinomycete family as a whole. Mycolic acids may be solvent-extracted from *C. pseudotuberculosis* without impairing the viability of the organism (Carne *et al.*, 1956).

The so-called “chemotaxonomic” approach to the classification of bacteria, based on analysis of the chemical composition of the cell wall, was at one time used relatively commonly. Analysis of cell wall mycolic acids greatly aided clarification of the taxonomy of actinomycetes, especially those of the genera Corynebacterium, Mycobacterium,
Rhodococcus and Nocardia (Minnikin et al., 1975; Goodfellow et al., 1976; Keddie and Cure, 1977; Minnikin and Goodfellow, 1980; Collins et al., 1982). In these studies, thin-layer (pyrolysis) chromatographic analysis of mycolic acid revealed that fatty acid chain length varied according to the genus and to a lesser extent the species. It was shown that mycobacterial mycolic acids normally consist of chain lengths of between 60 and 90 C$_{14}$ atoms, and may possess a number of distinct functional groups (Minnikin et al., 1978). In contrast, nocardiae and rhodococci were shown to possess shorter mycolic acids, consisting of between 36 and 66 C$_{14}$ atoms, and with fewer functional groups (Minnikin and Goodfellow, 1976). The mycolic acids of corynebacteria were found to be even smaller, being between 20 and 36 C$_{14}$ atoms in length, and usually saturated or containing a single double bond (Minnikin et al., 1978). Collins et al. (1982) reported that, consistent with the mycolic acid classification of corynebacteria, strains of C. pseudotuberculosis possessed mycolic acids with carbon chain lengths of between C$_{26}$ and C$_{36}$, which contained predominantly saturated C$_{14}$ side-chains (Collins et al., 1982).

There are few published data on the resistance of C. pseudotuberculosis to chemical disinfectants. However, most common disinfectants, including calcium hypochlorite, formalin and cresol solution, appear to be effective in killing the organism, but the presence of organic material necessitates increased exposure time (Ismail and Hamid, 1972). This protective effect is clearly significant for a pathogen commonly found within a thick matrix of purulent debris. The organism is capable of surviving in commercial sheep dip solutions for 24 h or more, a point of relevance to disease control (Nairn and Robertson, 1974).
Early reports showed that *C. pseudotuberculosis* isolates from different mammalian species shared identical biochemical characteristics, with the exception of nitrate reduction. Thus, the majority of isolates from horses and cattle reduced nitrate to nitrite, while those from sheep and goats did not (Knight, 1969; Sutherland et al., 1996). This led to the proposal of two distinct biotypes or subspecies (Biberstein *et al*., 1971). Based on this property of nitrate reduction, Songer *et al.* (1988) proposed the designations *C. pseudotuberculosis* biovar *ovis* (biotype 1) and *C. pseudotuberculosis* biovar *equi* (biotype 2). However, the isolation in recent years of nitrate-negative strains of *C. pseudotuberculosis* from cattle (Yeruham *et al*., 1997) and from horses (Connor *et al*., 2000) suggests that such categorization may be unsatisfactory.

A further minor difference between certain isolates lies in the area of antibiotic sensitivity. In a comparison of susceptibility to 17 different antimicrobial agents, the minimum inhibitory concentration of amikacin was higher for nitrate-negative sheep and goat isolates than for nitrate-positive equine and bovine isolates (Costa *et al*., 1998); however, the significance of this finding is not clear.

### 1.3.2. Virulence Factors:

No avirulent strain of *C. pseudotuberculosis* has yet been described; however, the organisms virulence mechanisms remain poorly understood. Since no plasmids have been identified in isolates of *C. pseudotuberculosis*, the absence of plasmid-encoded virulence determinants must be assumed. To date, research has focussed mainly on two known virulence factors identified as phospholipase D and mycolic acids. The genome of *C. pseudotuberculosis*, unlike that of a number of other bacterial pathogens, has yet to be fully sequenced; as a result, there
is at present no opportunity to identify novel gene sequences that may encode other virulence factors.

1.3.2.1. Phospholipase D:

PLD in this organism was first characterized by Carne (1940) and has since been detected in every isolate of *C. pseudotuberculosis* that has been studied, including isolates of both of the suggested biotypes, and all known strains of the organism recovered from infected mammalian species (Songer *et al.*, 1988). The contention that PLD represents a significant virulence factor is supported by much experimental evidence. Isolates of *C. pseudotuberculosis* in which the *pld* gene, encoding PLD, has been deleted from the chromosome or rendered inactive by mutation are incapable of causing the classic lymph node abscesses of CLA in sheep (Hodgson *et al.*, 1992; McNamara *et al.*, 1994). Similarly, the presence of specific antibody to PLD greatly limits the progress of the clinical disease.

PLD is defined as a sphingomyelin-specific phospholipase that catalyses the dissociation of sphingomyelin into ceramide phosphate and choline (Bernheimer *et al.*, 1980; Pepin *et al.*, 1994a). The *C. pseudotuberculosis pld* gene has been cloned and sequenced. Analysis reveals that it encodes a protein of some 31.4 kDa, preceded by a probable secretory signal sequence of 2.7 kDa (Hodgson *et al.*, 1990). The relatively large size of the protein molecule assists in its purification in the laboratory and enables large quantities to be collected (Egen *et al.*, 1989). Several biological activities have been reported for PLD, including dermonecrosis (Carne, 1940; Muckle and Gyles, 1986), lethality (Brogden and Engen, 1990), synergistic lysis of erythrocytes in the presence of an extracellular *Rhodococcus equi* factor (Fraser, 1961), and
inhibition of staphylococcal lysin-induced lysis of erythrocytes (Zaki, 1976); the two latter activities are employed as laboratory tests for the identification of *C. pseudotuberculosis*. PLD also interferes with ovine neutrophil chemotaxis and is lethal to the cells themselves (Yozwiak and Songer, 1993). In terms of the significance of PLD as a virulence factor, the activity that has been the focus of most interest is the increase in vascular endothelial membrane permeability engendered by the hydrolysis of sphingomyelin. This increased permeability leads to the leakage of plasma from blood vessels and into the surrounding tissues, and from there into the lymphatic drainage (Jolly, 1965; Carne and Onon, 1978). This effect may assist pathogenesis by favouring the lymphatic drainage of *C. pseudotuberculosis* in tissue fluid (Batey, 1986c).

PLD may assist the organism at the site of initial infection in other ways. It is known to activate the complementary pathway of the innate immune system, thereby depleting complement in the region surrounding the invading bacteria and protecting them from opsonization (Yozwiak and Songer, 1993). It may also impair the chemotaxis of neutrophils and, as a consequence, decrease the likelihood of phagocytosis early in infection (Yozwiak and Songer, 1993). In some respects this suggestion is at odds with other theories of pathogenesis, which propose that in the early stages of disease the organism parasitizes phagocytic cells and multiplies within them. Indeed, other authors have indicated that PLD may play a role in the escape of the bacterium from within macrophages. It is possible that this role is related to the action of PLD on the inner phospholipid layers of the macrophage cell membrane, as indicated by comparable observations on other bacterial infections (Titball, 1993).
1.3.2.2. Exotoxins:

Exotoxins with similarities to the PLD of *C. pseudotuberculosis* are known to be important virulence factors for other bacterial pathogens. For example, there is 97% homology between *C. pseudotuberculosis* PLD and the active exotoxin of *C. ulcerans*, a rare cause of human diphtheria (McNamara *et al.*, 1995). A PLD-like exotoxin is also considered to be an important virulence factor in *Pseudomonas aeruginosa* (Wilderman *et al.*, 2001). Likewise, *pld* shows homology to the *ymt* gene from the plague-causing organism *Yersinia pestis* (Hinnebusch *et al.*, 2000). PLD also demonstrates an intriguing similarity in structure and biological activity to an enzyme toxin produced by the venomous North American brown recluse spider, *Loxosceles reclusa* (Bernheimer *et al.*, 1985). This toxin induces dermonecrosis, haemolysis, platelet-aggregation and, on rare occasions, fatal renal failure (Lee and Lynch, 2005). Similarly, Hsu *et al.* (1985) showed that severe haemolytic crises resulted from the injection of *C. pseudotuberculosis* culture supernates or crudely purified exotoxin preparations into small ruminants.

1.3.2.3. Mycolic acid:

*Corynebacterium pseudotuberculosis* does not produce a protective capsule but has instead a waxy mycolic acid coat on the cell wall surface (described above). This coat has well-established cytotoxic properties, which play a major role in pathogenicity (Hard, 1972; Muckle and Giles, 1983; Tashjian and Campbell, 1983). The subcutaneous injection into mice of mycolic acid extracted from *C. pseudotuberculosis* results in the production of a localized swelling, with congestion and a central area of haemorrhagic necrosis. In addition, mycolic acid induces degenerative changes and death in phagocytizing leucocytes (Carne *et al.*, 2001).
1956). However, unlike the lethal effect of injection of similar molecules extracted from mycobacteria, the cytotoxic effect of *C. pseudotuberculosis* mycolic acid is confined to the site of injection (Hard, 1975).

Some authors have suggested that the mycolic acid coat enables *C. pseudotuberculosis* to survive for extended periods within the environment, a feature common to other members of the actinomycete family. *C. pseudotuberculosis* is indeed relatively resistant to environmental conditions (West *et al.*, 2002). Low ambient temperatures and mixing with particulate fomites enhance survival of the organism in discharged pus, viable bacteria being recoverable from inanimate surfaces for up to 55 days after contamination (Augustine and Renshaw, 1986). Batey (1986c) reported that under cold and damp conditions, the organism may remain viable in a farm environment for 6 months or more. Soil experimentally contaminated with pus still contained viable bacteria 8 months later (Brown and Olander, 1987). Thus, the environment may remain infectious for a significant period after contamination by an affected sheep.

In natural infections, the waxy mycolic acid coat of *C. pseudotuberculosis* provides the organism with mechanical, and possibly biochemical, protection from the hydrolytic enzymes present within lysosomes. This in turn enables the bacterium to survive phagocytosis and to exist within the host as a facultative intracellular parasite (Williamson, 2001). This capacity is likely to be essential for the migration of the organism from the point of initial entry to the eventual site of lesion development. In addition, the toxic nature of mycolic acid seems to contribute to abscess formation. In artificial infections of mice, a direct relationship was demonstrated between the quantity of cell wall
lipid produced by different isolates of *C. pseudotuberculosis* and their ability to produce chronic abscessation (Muckle and Gyles, 1983).

1.3.2.4. Other virulence factors:

A previously unreported 40 kDa protein antigen of *C. pseudotuberculosis*, suggested by Walker *et al.* (1994) to be a virulence factor, has yet to receive detailed study.

1.3.2.5. Other secondary factors:

Undetermined differences in virulence factors have been suggested to account for the specific “ovine/caprine” and “equine/bovine” strains of *C. pseudotuberculosis*, which are in other respects (cultural, antigenic and toxigenic) equivalent (Valli and Parry, 1993). The fact that the equine disease syndromes associated with the organism appear to be absent from large parts of the world in which the ovine infection is endemic, supports the existence of such differences. As discussed later, the probable importance of secondary factors, such as insect vectors, in *C. pseudotuberculosis* infections of horses may go some way to explaining this. However, in order to clarify the matter experimental infections with ovine and equine strains in parallel would be required.

1.4. Clinical and pathological features of caseous lymphadenitis in sheep:

*Corynebacterium pseudotuberculosis* infections in sheep are classically associated with the formation of pyogranulomas (Valli and Parry, 1993), and this accounts for the name “caseous lymphadenitis”. The lesions occur in two main forms, namely external (also known as superficial or cutaneous) and visceral, which may co-exist within the
same animal. The external form is characterized by abscessation of those lymph nodes that may be palpated externally. Any of the superficial lymph nodes of the body may be affected, dependent upon the original point of entry of the organism. Less commonly, localized purulent lesions not directly associated with the superficial lymph nodes may occur within the subcutaneous tissues. Such lesions may appear as organized abscesses, with swelling, fibrous encapsulation, loss of overlying hair and eventual rupture, resulting in the discharge of pus (Radostits et al., 2000).

The visceral form is associated with abscesses in the internal lymph nodes and other organs. In sheep, the principal location of these internal CLA lesions is the lung parenchyma and mediastinal lymph nodes. Lesions may also be found in the liver, kidneys or udder, and more rarely the heart, testis, scrotum, uterus, joints, brain or spinal cord (Valli and Parry, 1993).

1.4.1. Pathogenesis in sheep:

There is a general consensus among recent reviewers regarding the stages through which C. pseudotuberculosis infection progresses. After initial entry, the organism spreads rapidly to the local drainage lymph node. Here, multiple microscopic pyogranulomas develop, growing in size and coalescing to form larger abscesses. This is sometimes followed by a further extension of infection via the blood or the lymphatic system, leading to similar lesions in other organs. The nature of these slowly developing CLA lesions means that chronic, and frequently lifelong, disease is the rule rather than the exception. Viable bacteria may be recovered from abscesses several years after initial infection. Reactivation of disease may also occur, with the development of lesions at new sites after a considerable period of apparent quiescence.
Numerous routes of inoculation have been used to induce experimental CLA in sheep; intradermal, subcutaneous, intravenous, intratracheal, intravaginal, and intralymphatic inoculation have all proved successful in establishing disease (Nagy, 1976; Burrell, 1978a; Pepin et al., 1994b; Fontaine et al., 2006). In natural infections, however, the principal route of entry is believed to be through the skin (Batey, 1986c; Brown and Olander, 1987; Davis, 1990; Collett et al., 1994). This initial infection is facilitated by minor cutaneous wounds and abrasions, especially those caused by shearing (Paton et al., 1988). Wounds caused during castration or docking have also been suggested as an occasional route of entry, as has the umbilicus in neonatal animals (Valli and Parry, 1993). Entry via the oral cavity has been postulated to account for the small number of head and neck lesions seen in sheep from the Antipodes and North America, and the many more such lesions in goats (Ashfaq and Campbell, 1979). In contrast, the more distal parts of the intestinal tract are not believed to provide a portal of entry for the organism, even in the presence of parasitic damage (Valli and Parry, 1993).

A respiratory route of infection, postulated by Stoops et al. (1984), has been widely quoted in subsequent reviews. This theory was based on the observations that some naturally infected sheep show only pulmonary lesions, and that a small number of these lesions are located within the walls of airways. Moreover, Brown and Olander (1987) reported the production of disseminated pulmonary abscesses by injecting intratracheally a broth culture of *C. pseudotuberculosis*. However, other studies have indicated that such pulmonary lesions may develop as part of a systemic infection initiated elsewhere in the body. Thus, after the intravenous inoculation of lambs with *C. pseudotuberculosis*, the majority of internal lesions appeared in the lungs and associated thoracic lymph
nodes (Brogden et al., 1984). It has also been noted that in natural ovine CLA infections, the patterns of distribution of pulmonary lesions are consistent with haematogenous or lymphogenous spread rather than with aerogenous spread (Nairn and Robertson, 1974). It would therefore appear that entry of infection via the respiratory tract, although a theoretical risk, is of minor importance.

The use of radioisotopically labelled inflammatory cells and scintigraphic imagery demonstrated that, within a few hours of subcutaneous inoculation with bacteria, huge numbers of neutrophils were recruited to the injection site, and by 24 h post-inoculation, these neutrophils began to appear in the local drainage lymph node (Pepin et al., 1992); the relative importance of neutrophils decreased from day 3, while the relative numbers of macrophages at the site of inoculation rose dramatically. As suggested above, the ability of C. pseudotuberculosis to survive phagocytosis by such cells and to exist as a facultatively intracellular parasite enables the organism to be carried within these cells via the lymphatic drainage to the local lymph node (Pepin et al., 1994a). Thus, phagocytic cells recruited to the area in response to infection become the means by which further colonization of the body is brought about.

Once a lymph node has been colonized by C. pseudotuberculosis it undergoes a short period of generalized inflammation. PLD, the soluble exotoxin produced by C. pseudotuberculosis, is the probable initiator of this lymphadenitis. Pepin et al. (1991) reported that within 24 h of the subcutaneous inoculation of lambs a number of micro-abscesses occurred within the cortical region of the lymph node draining the site of inoculation. By day six post-inoculation, these micro-abscesses had become more numerous and began to expand and coalesce to form larger
purulent foci. The early pyogranulomas contained clumps of bacteria and cellular debris, with a relatively high proportion of eosinophils, giving the purulent core a slightly green hue. At the same time, and in parallel with the cellular events at the point of entry, the infiltration of neutrophils diminished and monocytes/macrophages became the predominant cell type within the lesion (Pepin et al., 1994b). A process during which the lesion is encapsulated followed shortly thereafter, leading to a diminution of the inflammatory reaction in the parenchyma of the node. Continued slow expansion of the lesion sometimes then occurred, depending on the location of the node and whether or not it ruptured to discharge its contents. Such enlargement progressed through a repeated cycle of necrosis and reformation of the outer capsule. In the early stages, the purulent contents of the abscess were soft and semi-fluid; however, as time progressed, the pus within the lesion took on a more plastic or solid form, in which scattered clumps of bacteria were sometimes noted. Small nodules of mineralization formed within the purulent material, causing it to become progressively paler in colour. These calcified foci tended to be laid down in concentric layers reminiscent of the cross-sectional view of an onion. This classic “onion ring” presentation is regarded as virtually pathognomonic for CLA. Superficial lymph node abscesses may expand to reach a diameter of as much as 15 cm, but 3–5 cm is a more common size (Valli and Parry, 1993).

It is likely that the infected superficial lymph node acts as staging post for the further colonization of internal lymph nodes and viscera; however, at which stage in the course of the infection such colonization occurs is the subject of some speculation. It seems probable that purulent emboli detach from pyogranulomas within the affected nodes, pass into the efferent lymphatic flow and are then delivered into the bloodstream.
(Radostits et al., 2000). However, once encapsulation of a lymph node abscess has taken place, the escape of such infectious emboli seems likely to occur infrequently, if at all. Consequently, it has been argued that in most cases any colonization of organs beyond the local drainage lymph node must occur relatively quickly after initial infection, and that phagocytic cells may once again be the vehicles (Pepin et al., 1994a).

Other than the lymph nodes, the lung is the most frequent site of CLA lesions. As already noted, the generally random distribution of lesions within the parenchyma is consistent with haematogenous or lymphogenous spread, with seeded lesions developing from within the alveolar vasculature (Batey, 1986c). These pulmonary lesions most commonly take the form of encapsulated abscesses similar to those seen in the lymph nodes, but occasionally a more extensive bronchopneumonia is also recorded (Valli and Parry, 1993). The latter may lead to areas of pleuritis, resulting in the development of fibrinous or fibrous adhesions to the chest wall, pericardium or diaphragm (Renshaw et al., 1979; Stoops et al., 1984). In animals with pulmonary abscesses, CLA lesions are occasionally encountered in the associated mediastinal and bronchial lymph nodes, implying an additional step in the transit of the organism from the lung parenchyma. Abscesses within the mediastinal lymph nodes may become so large as to put pressure on the adjacent oesophagus, interfering with normal swallowing and rumination, and leading to chronic ill-thrift (Paton et al., 2005).

Mastitis due to C. pseudotuberculosis is encountered occasionally in sheep and is most likely to represent an extension of infection from the adjacent supra-mammary lymph node. It may take the form of an acute suppurative mastitis, or appear as chronic encapsulated abscesses within the mammary gland. Lesions in other organs such as the liver, kidneys
and scrotum also tend to be encapsulated abscesses containing a thick caseous material, which may again take on a lamellated form in chronic cases (Valli and Parry, 1993). On rare occasions, the organism has also been isolated from the stomach contents and tissues of ovine fetuses, consistent with a role in abortion (Dennis and Bamford, 1966).

1.5. Control and eradication of disease in sheep:

1.5.1. Diagnosis:

For the successful control of CLA, it is first necessary to identify infected animals, so that they can be prevented from coming into contact with uninfected animals. The diagnostic criterion for CLA is the culture and identification of \textit{C. pseudotuberculosis}. It is usually possible to isolate the organism from lesions of all ages, although the number of viable bacteria present in chronic abscesses may be low and apparently sterile lesions are occasionally encountered. In sheep, the presence of abscesses in external lymph nodes is highly suggestive of the disease, particularly if several animals in a group are similarly affected. Other bacterial pathogens, such as \textit{Actinobacillus licheniformis}, \textit{Arcanobacterium pyogenes}, and in some countries \textit{Staphylococcus aureus} subsp. \textit{anaerobius}, are all capable of producing suppurative lymphadenopathy (Bek-Pederson, 1997; Pekelder, 2000); however, these other infections tend to be sporadic in nature and are rarely seen as a flock problem.

In the past, identification of \textit{Corynebacterium} spp. has proved difficult for several reasons. These reasons include insufficient numbers of reference strains with which to compare isolates, inadequacies in the reference data obtained from characterized strains, and the relative lack of
appropriate criteria, (Hill et al., 1978). It has long been considered that *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans* are related, as they share a similar morphology and cell wall composition, and all produce a characteristic halo on Tinsdale medium, indicating the production of a cystinase enzyme (Barksdale, 1970). However, on the basis of DNA homology, there would seem to be less similarity between the three species than was at one time thought (Groman et al., 1984). Nonetheless, *C. pseudotuberculosis* and *C. ulcerans* can be made to produce diphtheria toxin by infecting them with toxin-associated phages from *C. diphtheriae* (Saxholm, 1951; Henriksen and Grelland, 1952; Maximescu, 1968).

In the laboratory, *C. pseudotuberculosis* cultured from clinical samples may be identified from its enzymatic profile and its ability to utilize various carbohydrate sources. The task of biochemical profiling has been greatly simplified by the introduction of standardized and miniaturized proprietary test kits, an example of which is the Analytical Profile Index (API) identification system (bioMérieux [UK], Basingstoke, Hampshire, UK). The “API Coryne” kit (used for the identification of coryneform bacteria) comprises 21 individual test substrates for the determination of enzymatic activity or carbohydrate fermentation. After inoculation with the test organism and incubation for a defined period, the metabolic end-products of the enzymatic tests result in the development of specific colour changes, either spontaneously or following the addition of reagents. For substrate fermentation tests, pH change is also detected colorimetrically. Subsequently, a particular combination of positive and negative test results is used to compile a numerical profile (an Analytical Profile Index; API), which is then used to “interrogate” a proprietary software database.
In addition to substrate utilization, further laboratory-based tests are available to identify *C. pseudotuberculosis*. Some years ago, it was noted that when β-lysin-producing staphylococci were cultured on blood-containing solid media in the presence of strains of *Streptococcus agalactiae* that produce a secreted ‘factor’, enhanced zones of haemolysis were observed. This led to the discovery of an *S. agalactiae* protein, designated CAMP-factor, which on its own was unable to cause erythrocyte lysis. The synergistic lysis phenomenon was designated the CAMP-reaction (*Christie et al.*, 1944). Similarly, when *C. pseudotuberculosis* and *Rhodococcus equi* are cultured together on solid blood-containing media, colonies of both species in close proximity to each other cause significant zones of erythrocyte lysis (*Fraser, 1961*). A similar phenomenon was noted when *C. pseudotuberculosis* was cultured in the presence of δ-lysin-producing staphylococci (*Lovell and Zaki, 1966*). The *R. equi* protein (so-called equi factor) involved in synergistic lysis was found to be a phospholipase C enzyme (*Bernheimer et al.*, 1980). Currently this assay, which may also be performed by supplementing blood-containing solid media with *R. equi* culture supernate (containing equi factor), is used in diagnostic laboratories for the identification of *C. pseudotuberculosis*. However, it should be noted that *C. ulcerans* also produces PLD, although significantly it is the only other corynebacterial species found to do so (*Barksdale et al.*, 1981). Hence, PLD activity is a distinctive marker within the genus *Corynebacterium*.

### 1.5.2. Treatment:

*In vitro*, *C. pseudotuberculosis* is sensitive to a range of antibiotic chemicals. In standard laboratory-based tests, most commonly used antibiotic classes have been shown to prevent bacterial growth and
multiplication (Muckle and Gyles, 1982; Judson and Songer, 1991). However, in vivo, clinical CLA is generally refractory to antibiotic therapy, probably because of the thick encapsulation around the typical lesions, and the thick and caseous nature of the pus contained within (Williamson, 2001). The intracellular nature of the organism during parts of the disease cycle is also believed to confer some protection from certain commonly employed antibiotics. Surgical treatment of external lesions has been suggested as an alternative to culling in the case of particularly valuable animals (Davis, 1990). Whether the lesion is surgically removed, or simply lanced and flushed-out daily until healed, parenteral antibiotic treatment for 4–6 weeks has been recommended to reduce the likelihood of recurrence. This course of action is still seen as unreliable at best, since it relies upon the antibiotic to remove all infecting organisms from the treated lesions, and assumes that no internal lesions are present. As a result, reports on the use of such techniques are not encouraging (Gezon et al., 1991; Rizvi et al., 1997).

Significantly, in a recent report claims were made of successful treatment of C. pseudotuberculosis-infected sheep with a combination of the antibiotics rifamycin and oxytetracycline (Senturk and Temizel, 2006). Rifamycin is primarily used against Mycobacterium tuberculosis and Mycobacterium lepraе infections, and has an established ability to kill susceptible intracellular bacteria. In small trial, 10 CLA-affected animals received twice-daily treatment with rifamycin for a period of 10 days, in association with injections of a depot formulation of oxytetracycline at intervals of 3 days. This led to a decrease in the size of CLA lesions in the external lymph nodes of affected sheep, producing what was described as a clinical resolution of the lesions. However, no necropsies followed the intensive treatment regime and the report did not
indicate for how long the sheep remained disease-free. Further studies are required before treatment of CLA cases becomes a viable alternative to culling (Baird, 2006).

1.5.3. Serology:

Whilst isolation and identification of *C. pseudotuberculosis* remains the gold standard in diagnosis of CLA, this may not always be advantageous or possible. The puncture of CLA abscesses generally releases pus on to the animal's skin or into the environment, presenting a risk of transmission of infection to other animals. Furthermore, chronic external lesions that have ruptured frequently become fibrosed and may contain little pus and few viable organisms. Finally, animals suffering from the visceral form of disease may show no external lesions that can be sampled, but remain a potential source of infection to others. Much research interest has therefore focussed on serological tests that might identify CLA without recourse to bacteriology.

Most serological diagnostic tests for CLA are based on the detection of a humoral response to PLD exotoxin. Such tests have been explored as a method for controlling the disease in sheep by identifying and removing infected carrier animals. The use of serological tests for the laboratory diagnosis of *C. pseudotuberculosis* infection dates from the early years of the 20th century. Originally, determination of infection status relied upon the invivo demonstration of antitoxins (Forgeot and Cesari, 1912; Nicolle *et al*., 1912; Watson, 1920; Mitchell and Walker, 1944; Doty *et al*., 1964; Zaki, 1976). Experimental subjects (mice, rabbits or guinea-pigs) were given injections of serum from animals suspected of being infected with *C. pseudotuberculosis*; they were then given lethal doses of PLD toxin, prepared from *C. pseudotuberculosis* culture
supernates. A reduction in the rate of mortality of serum-treated animals, as compared with that of controls, was considered to indicate passive protection by serum antitoxin, and to be highly suggestive of *C. pseudotuberculosis* infection.

In a development that was to facilitate invitro serological screening, it was found that inclusion of antitoxin-containing sera in the CAMP-inhibition test resulted in neutralization of the inhibitory effect that *C. pseudotuberculosis* exerted on staphylococcal \( \beta \)-lysin; this led to a new method designated the anti-haemolysin-inhibition (AHI) test for testing sera from animals suspected of having CLA. This test has been used for disease diagnosis in sheep, goats and horses, avoiding the use of experimental animals (Zaki, 1968; Knight, 1978; Burrell, 1980; Brown *et al.*, 1986).

Other tests used in the study of CLA include tube agglutination assays (Cameron *et al.*, 1972; Husband and Watson, 1977), an indirect haemagglutination test (Shigidi, 1978) and a double immunodiffusion test (Burrell, 1980). However, the enzyme-linked immunosorbent assay (ELISA) for use in diagnosis has shown particular promise. Initially CLA-diagnostic ELISAs employed crude *C. pseudotuberculosis* cell wall preparations or supernate-derived exotoxin as antigen (Maki *et al.*, 1985; Ellis *et al.*, 1990; Sting *et al.*, 1998). Test sensitivity was usually regarded as good, but specificity was relatively poor—possibly due to cross-reactions with other proteins present in the antigen preparations. However, some ELISAs have been claimed to be sufficiently specific and sensitive for field diagnosis. One such ELISA was based on a PLD antigen derived by recombinant technology from *E. coli* containing a plasmid bearing the *pld* gene (Menzies *et al.*, 1994), a test sensitivity of 86.3% and specificity of 82.1% being reported. The assay coming closest
to the requirements of a diagnostic test was reported by workers in the Netherlands (Ter Laak et al., 1992) and was later refined to improve specificity and sensitivity (Dercksen et al., 2000). This is an indirect double antibody sandwich ELISA, based on PLD exotoxin purified from the supernate of C. pseudotuberculosis culture, polyclonal rabbit anti-PLD serum being used as a capture antibody. The improved method is reported to have a specificity in sheep of 99±1%, and a sensitivity of 79±5%.

Other potential tests include a polymerase chain reaction (PCR) method (Cetinkaya et al., 2002) and a bovine interferon (IFN)-γ whole blood ELISA (Menzies et al., 2004). The latter was the first test in which assay of cellular immunity to C. pseudotuberculosis was used as a diagnostic tool. Over the course of 1 year, the IFN-γ test accurately detected goats experimentally infected with C. pseudotuberculosis with a reliability of 89.2%, and non-infected goats with a reliability of 97.1%. Over the same period, a recombinant PLD ELISA detected the experimentally infected goats with a reliability of 81.0% and non-infected goats with a reliability of 97.0%. The PLD ELISA was, however, more predictive than the IFN-γ ELISA of the lesions subsequently observed at necropsy. A later study of INF-γ production in cultures of whole blood compared infected goats following stimulation with either secreted bacterial antigen or somatic antigen; IFN-γ production by blood cells was found to be significantly higher in response to the secreted antigen (Meyer et al., 2005).
1.5.4. Vaccination:

1.5.4.1. Bacterin vaccines:

The earliest reported vaccination trials in sheep were conducted in the Patagonia region of Argentina, where a formalin-killed *C. pseudotuberculosis* vaccine reduced infection by up to 60% (Quevedo *et al.*, 1957). Cameron *et al.* (1972) made one of the first attempts to demonstrate by experimental challenge the ovine immune response to vaccination, the vaccine consisting of formalin-killed, whole cells of *C. pseudotuberculosis*. The intravenous challenge doses investigated varied from $2 \times 10^7$ to $2 \times 10^{10}$ colony-forming units. However, it was difficult to obtain satisfactory results, the higher doses producing acute death due to pulmonary oedema and the lower doses failing to cause lesions. Furthermore, vaccination produced increased titres of serum antibody that persisted for only 3–4 months. Nonetheless, it was concluded that vaccination could protect sheep against the lethal effects of sub-acute infection but not against formation of the lesions associated with chronic infection. It was considered that effective immunity might not depend solely on circulating antibody, but that cellular immune responses might play a role. It was also suggested that, if used, a whole cell vaccine should be administered shortly before shearing.

More recently, in a study of several vaccines, immunization of sheep with a formalin-killed, virulent, UK-derived *C. pseudotuberculosis* isolate and aluminium hydroxide adjuvant resulted in statistically significant protection against homologous challenge; the vaccine had prevented the spread of infection beyond the site of inoculation (Fontaine *et al.*, 2006).
There have been few investigations of the protective effect of *C. pseudotuberculosis* bacterins against disease in the field. Menzies *et al.* (1991) conducted a field trial of a killed whole-cell vaccine in a flock of sheep and herd of goats, both populations having shown a prevalence of CLA in adult animals ranging from 15% to 30% in the pre-trial period. During the trial, lambs and kids were vaccinated at 2.5–3.5 months of age, with two further doses administered at 1 month and 11 months after the initial vaccination. The animals were regularly monitored for 36 months after the initial dose of vaccine. Serum antibody concentrations were significantly increased in vaccinated sheep and goats for the full term of the experiment. Furthermore, vaccination significantly decreased the incidence of CLA in the sheep, and there was a suggestion of a similar effect in the goats.

In a later study, juvenile sheep and goats were vaccinated with a *C. pseudotuberculosis* bacterin formulated in light mineral oil containing muramyl dipeptide (Brogden *et al.*, 1995); the results, however, were inconclusive.

1.5.4.2. Toxoid vaccines:

Most subsequent studies on CLA vaccination focused on the use of bacterins, or on vaccines made from components of whole cells. However, a report by Jolly (1965) showed that the administration of toxoid, followed by toxin, reduced the extent of experimental CLA in sheep. This followed the observation by Cameron (1964) that an undefined “protoplasmonic toxin” of *C. pseudotuberculosis* played a role in the induction of protective immunity. It was later demonstrated that *C. pseudotuberculosis* exotoxin was in reality identical with this
protoplasmic toxin (Cameron and Smit, 1970), adding weight to the hypothesis that PLD could be used as a protective antigen.

Purely managemental strategies having failed to prevent the spread of the disease, studies were conducted in Australia during the 1970s to develop a CLA vaccine, work that would eventually lead to the first commercial CLA vaccine (Eggleton et al., 1991a). The ability of PLD to protect against CLA was reported by both Nairn et al. (1977) and Burrell (1978b).

Between 1977 and 1978, the technology required for production of an effective toxoid vaccine was made available to the Commonwealth Serum Laboratories (CSL; Parkville, Victoria, Australia). For vaccine production, PLD was obtained from filtered and concentrated *C. pseudotuberculosis* culture supernates, which were subsequently toxoided with formalin (Burrell, 1983; Hodgson et al., 1999). A combined clostridial and corynebacterial vaccine, known as Glanvac™, was released in 1983 (Eggleton et al., 1991); it subsequently became available in Australia and several other countries. In one report, vaccination of goats with Glanvac™ resulted in statistically significant protection against subsequent experimental infection with *C. pseudotuberculosis*, as measured by a reduction in the number of lesions (Brown et al., 1986). In addition, the results of trials performed in the process of the commercial development of Glanvac™ indicated significant protection of sheep against CLA (Burrell, 1983; and Eggleton et al., 1991a), however, found that PLD toxoid gave greater protection when not combined with clostridial components.

The mechanism by which Glanvac™ confers immunity has been the topic of some debate. It has been suggested that protection against
CLA is both humoral and cell-mediated, but that PLD is neutralized by circulating antibody (Burrell, 1978a; Sutherland et al., 1992). In contrast, Hodgson et al. (1999) discussed the possibility that the small amount (ca 1%) of PLD activity that remained after formalin treatment (Eggleton et al., 1991b) might be responsible for the protection conferred by Glanvac™. However, Eggleton et al. (1991b) had previously reported that a four-fold variation in the level of active toxin remaining in a toxoid vaccine “neither enhanced nor reduced the protective potency of the vaccines”. Others have reported that anti-exotoxin antibodies are not on their own sufficient to prevent infection in the absence of other (probably cell-mediated) immune defences (Irwin and Knight, 1975; Ellis et al., 1990). Significantly, Cameron and Smit (1970) stated that “…it could be expected that crude exotoxin preparations could also contain other antigens. Therefore, unless an absolutely pure preparation of exotoxin can be produced, its immunizing activity can never be accurately assessed”. This statement was later reiterated by Burrell (1983), who confirmed that the culture supernates used to prepare Glanvac™ contained other C. pseudotuberculosis somatic antigens, which varied in amount from batch to batch. Moreover, Walker et al. (1994) intermittently identified antibodies against a novel C. pseudotuberculosis antigen in sera from sheep vaccinated with Glanvac™. It would therefore seem that the presence of other C. pseudotuberculosis proteins in toxoid vaccines prepared from culture supernates may well contribute to the protective capacity of the vaccine.

1.5.4.3. Combined vaccines:

In reviewing CLA vaccine development, Burrell (1983) stated that, despite the capacity of cell-associated antigens and PLD (Burrell, 1978b) to give some protection, full protection required the presence of both. In
defence of this, it was revealed that an alhydrogel-adjuvanted vaccine composed of formalin-killed whole *C. pseudotuberculosis* cells, enriched with formalin-treated, PLD-rich culture supernate, protected completely against experimental *C. pseudotuberculosis* infection and outperformed vaccines consisting of cell-free toxoided culture supernates (Burrell, 1983). In contrast, a report of work conducted during the development of Glanvac™ revealed that supplementation of cell-free toxoid vaccine with *C. pseudotuberculosis* cells made no difference to the observed protection (Eggleton et al., 1991b).

A further commercially available CLA vaccine (Caseous D-T™; Colorado Serum Co., Denver, CO, USA) contains clostridial toxoids and a combination of *C. pseudotuberculosis* bacterin and toxoid. Piontkowski and Shivvers (1998) reported that this vaccine gave some protection to sheep against experimental infection, in that it reduced the incidence of external and internal CLA lesions.

Fontaine et al. (2006) reported that vaccination of sheep with a *C. pseudotuberculosis* bacterin enriched with recombinant PLD resulted in complete freedom from infection 3 weeks after experimental homologous challenge. It had been reported previously (Simmons et al., 1998) that *C. pseudotuberculosis* (even a non-virulent mutant) was capable of persisting within granulomas in the draining lymph node, as a result of the inaccessibility of abscesses to the immunological factors required to clear the infection. Therefore it was suggested that vaccination had enabled clearance of the organism prior to establishment within the lymph node.
1.5.3.4. Live vaccines:

Pepin et al. (1993) reported that sheep infected experimentally with *C. pseudotuberculosis* were protected against further challenge, despite being unable to clear the original infection. This observation indicates the possible value of an attenuated vaccine.

Because PLD is an important virulence factor (Carne and Onon, 1978), Hodgson et al. (1992) hypothesized that genetic inactivation of the pld gene might provide the basis for a live recombinant veterinary vaccine. A mutant derivative of pld was constructed *in vitro*, by cloning the gene into a suitable plasmid vector (pEP2), and disrupting it by insertion of an erythromycin-resistance cassette. Subsequently, the resulting recombinant plasmid (pBTB58) was introduced into *C. pseudotuberculosis* by electroporation, and recombination between homologous plasmid-borne and chromosomal sequences resulted in replacement of the wild-type gene by the mutant copy. To ensure the stability of the mutation, pBTB58 vector sequences were removed from mutant cells by transformation with the plasmid pEP3; because pEP3 possesses the same origin of replication as that of pBTB58, plasmid segregation resulted in loss of pBTB58 sequences and retention of pEP3.

Inoculation of sheep with up to $10^7$ colony-forming units of the PLD-deficient *C. pseudotuberculosis* strain (so-called “Toxminus”) failed to produce the abscessation normally associated with wild-type *C. pseudotuberculosis*. However, at higher doses, transient abscesses were formed at the inoculation site. Despite this, the authors concluded that the degree of attenuation was equivalent to at least a 100-fold reduction in infective dose. The vaccine potential of the attenuated strain was determined by challenge of Toxminus-immunized sheep with wild-type
C. pseudotuberculosis. A degree of protection against challenge and strong humoral and cellular immune responses were observed, but these effects were lower than those produced by wild-type infection.

It was later postulated (Hodgson et al., 1994) that the rationale behind the creation of the Toxminus mutant was flawed: while the recombinant strain was attenuated for virulence and hence useful as a live vaccine vector, one of the major immunodominant antigens (PLD) had been removed, thus reducing immune stimulation. Hodgson et al. (1994) therefore constructed a mutant derivative of PLD in which the histidine_{20} amino-acid residue (a constituent of the enzyme's active site) was substituted for tyrosine, and expression of the resulting plasmid-borne, mutant pld gene produced a biologically inactive derivative of PLD. On transformation of Toxminus with the inactivated pld construct, cells were able to secrete an inactive PLD derivative, albeit at a level lower than that of wild-type PLD.

In an attempt to avoid inoculation-site reactions and to provide a convenient means of vaccine administration, Hodgson et al. (1994) administered each of two vaccines (Toxminus and Toxminus carrying the inactivated pld) to sheep via the oral route. Both vaccines induced significant and similar humoral immune responses. Furthermore, in animals immunized with the strain expressing the inactivated pld, challenge elicited a rapid anamnestic response to PLD; this response was quicker and of greater magnitude than the humoral response (1) to infection in unvaccinated animals, or (2) in those receiving Toxminus alone. Despite significant humoral responses, the protection against challenge conferred by the Toxminus strain was negligible; this contrasted with a previous report of subcutaneous vaccination with Toxminus (Hodgson et al., 1992). The difference in protection was
attributed to the failure of oral vaccination, unlike subcutaneous vaccination, to stimulate a significant Th1 immune response, IgG1 having been identified as the dominant IgG isotype. Despite the failure of Toxminus alone, immunization with Toxminus expressing the mutant \( pld \) gene resulted in significant protection against challenge, confirming the earlier observations that antibodies against PLD protected against CLA (Jolly, 1965; Nairn et al., 1977; Eggleton et al., 1991c).

Continued efforts to produce an effective live vaccine vector led to attempts to express a range of foreign antigens in the Toxminus strain, under the control of several different promoters (Moore et al., 1999). In the course of this work, Toxminus-based systems were developed to enable antigens to be expressed, intracellularly or in secreted form, as native or fusion proteins, either constitutively or by controlled induction. Furthermore, genes were successfully expressed from plasmids, or following integration into the Toxminus chromosome. In addition to derivatives of \( C. \) pseudotuberculosis \( pld \), genes from other pathogenic organisms, including Mycobacterium leprae, Taenia ovis, Babesia bovis, Dichelobacter nodosus and Anaplasma marginale, were successfully expressed. Subsequently, it was revealed that subcutaneous immunization of sheep with Toxminus expressing inactive \( pld \), or \( A. \) marginale or \( D. \) nodosus antigens, resulted in the induction of a significant humoral immune response; the same was not true, however, for \( T. \) ovis or \( B. \) bovis antigens.

1.5.4.5. DNA vaccines:

With the advent of DNA vaccination technologies, there have been reports of the successful immunization of numerous species against various micro-organisms (Donnelly et al., 1995), but such reports in
respect of farm livestock have been relatively few in number (Van Drunen Littel-van den Hurk *et al*., 2000). In general, DNA vaccines would seem to have been less successful than conventional or sub-unit protein vaccines in protecting against infection, probably due to a weak and short-lived immune response (Rothel *et al*., 1997; Schrijver *et al*., 1997).

In an effort to improve the efficacy of DNA vaccination against intracellular pathogens, fusion of antigen-encoding genes to those encoding specific “effector” molecules has been attempted. One such effector molecule is the cytotoxic T lymphocyte antigen-4 (CTLA-4) (Brunet *et al*., 1987), which assists in directing secreted vaccine antigens to lymph nodes and antigen-presenting cells (APCs) (Boyle *et al*., 1998; Drew *et al*., 2001). CTLA-4, a repressor of T-lymphocyte proliferation, is expressed on the surface of activated T cells. In its dimeric form it competes with CD28 for binding to the CD80 and CD86 (B7 domains) on antigen-presenting cells (Linsley *et al*., 1991; Thompson and Allison, 1997). A truncated derivative of CTLA-4 (designated CTLA-4-HIg) was created, composed of the C-terminal end of the extracellular domain fused to the hinge CH2 and CH3 domains of human IgG1 (HIg); this derivative was able to dimerize as a result of the IgG hinge domains. Subsequently, chimeric proteins, consisting of antigen-encoding sequences fused to CTLA-4-HIg, were found to be specifically targeted to APCs (Boyle *et al*., 1998). In a later study it was shown, in vaccinated mice, that the antibody response to a *T. ovis* 45W antigen-CTLA-4-HIg fusion construct was 30 times higher than that achieved with non-targeted DNA vaccination. Furthermore, the kinetics of the antibody response to the targeted construct were faster than achieved with non-targeted DNA or with an adjuvanted protein vaccine; unfortunately, vaccination of
outbred sheep with the fusion vaccine failed to enhance immune responses (Drew et al., 2001).

In the only report of the development of a DNA vaccine against *C. pseudotuberculosis* infection, De Rose et al. (2002) fused the sequence encoding the extracellular domain of bovine CTLA-4 (Parsons et al., 1996) with that encoding the CH2 and CH3 domains of human IgG1 (Boyle et al., 1998) and that of a genetically inactivated derivative of PLD (∆PLD) (Tachedjian et al., 1995), to generate the construct boCTLA-4-Ig-∆PLD. In an attempt to determine the optimal route of immunization, the DNA vaccine was administered either intramuscularly in the left thigh, subcutaneously in the groin, or via gene gun delivery into the skin of the lower rear flank. Subsequently, antibody responses and resistance to experimental challenge with *C. pseudotuberculosis* were examined. Before challenge, only intramuscular vaccination resulted in a significant (albeit minimal) increase in serum anti-∆PLD IgG concentrations. After challenge, antibody concentrations in animals vaccinated by the gene gun or subcutaneous method were not significantly higher than in non-vaccinated animals; however, IgG concentrations in intramuscularly vaccinated animals increased dramatically after challenge, suggesting a strong anamnestic response. Furthermore, sterile immunity (i.e., absolute protection from infection) was observed in 45% of animals vaccinated intramuscularly, which was similar to the results of a previous study (Hodgson et al., 1999) of vaccination with ∆PLD protein. IgG2 titres in animals vaccinated intramuscularly were significantly elevated, suggesting that a Th1 immune response was responsible for the observed protection against infection.
1.5.5. Identification of immunodominant antigens:

In attempts to develop more refined CLA vaccines, PLD has been the main focus of research. This was due, in part, to the perceived importance of the toxin in the dissemination of *C. pseudotuberculosis* within the infected host (Carne and Onon, 1978) and to the observation that anti-PLD antibodies assist in clearance of *C. pseudotuberculosis* from experimentally infected animals (Eggleton *et al*., 1991). It is also probable that, in the absence of a *C. pseudotuberculosis* genome sequence and of information on other virulence determinants, PLD was favoured for inclusion in novel vaccines. However, as discussed above, *C. pseudotuberculosis* cell-associated antigens also contribute to protection against CLA (Brogden *et al*., 1990; Hodgson *et al*., 1992), and have even been suggested to be essential for the induction of optimal immunity (Cameron *et al*., 1969; Cameron, 1972; Burrell, 1983).

In an investigation of immunodominant *C. pseudotuberculosis* antigens (Muckle *et al*., 1992), the presence of seven immunodominant proteins in whole-cell lysates of *C. pseudotuberculosis* was investigated with immune sera from goats with CLA. Of these seven proteins, three were consistently detected by all sera. Sodium chloride extracts of *C. pseudotuberculosis* contained all three immunodominant antigens, suggesting that they were loosely associated with the bacterial cell wall. Subsequent purification and functional analysis of the most strongly immunoreactive protein identified it as PLD; however, the identity of the two remaining antigens remained undetermined.

In a later study, Walker *et al.* (1994) adopted a novel approach to the identification of immunodominant *C. pseudotuberculosis* antigens based on B lymphocytes derived from infected loci in sheep. Such
antibody-secreting cells (ASCs) had previously been shown to produce antibodies of highly restricted specificity (Meeusen and Brandon, 2006). After experimental infection of sheep with *C. pseudotuberculosis* in the right hock, bacteria drained to popliteal lymph nodes, from which site ASCs were subsequently obtained. The popliteal lymph nodes were dissected and mononuclear leucocytes were isolated. These leucocytes were cultured in the presence of mitogens, and the culture supernates were referred to as ASC probes. Immunoblots of *C. pseudotuberculosis* whole-cell lysates were performed with the ASC probes. A previously undescribed *ca* 40 kDa antigen was consistently and strongly recognized by the probes derived from all experimentally infected animals. The 40 kDa antigen, which was present in *C. pseudotuberculosis* culture supernate, was subsequently purified by chromatography. Immunization of sheep with 100 µg of the purified antigen (later found to be a serine protease designated CP40 [Wilson *et al.*, 1995]), resulted in *ca* 79% protection against experimental *C. pseudotuberculosis* infection. Vaccination with 5 µg of the antigen, although not inducing complete protection, significantly reduced the number of lung lesions in infected sheep.

The results obtained through use of ASC probes were significant, since they revealed that the CP40 antigen, which was found in all *C. pseudotuberculosis* strains examined, was one of the earliest products to be recognized by the host during infection (Walker *et al.*, 1994). There was no obvious relation between CP40 antibody titres and the amount of antigen administered or incidence of lung lesions, implying that cell-mediated immunity was the main protective mechanism.
Chapter two
Materials and Methods

2.1. Sterilization:

2.1.1. Hot-air oven:

Glassware like Petri dishes, pipettes, tubes, flasks and glass rods were sterilized in the hot air oven at 160°C for 1 hour.

2.1.2. Autoclaving:

Sterilization of media, solutions, screw capped bottles; rubber-stopper, etc were sterilized by autoclaving at 121°C (151b/square inch) for 15 minutes and 110°C (10 lb/square inch) for 5 minutes for sugar media.

2.2. Collection of blood and serum:

Blood and serum used for the enrichment of media were collected by veni-puncture of the jugular vein of a healthy donor sheep. Blood obtained in sterile flasks with glass beads was defibrinated by shaking, and used for preparation of blood agar, and that needed for serum preparation was allowed to clot at room temperature for one hour, clot was separated from the walls of the container by using sterile glass rod, which were then transferred to refrigerator at 4°C and left overnight. Serum which collected was pipette into centrifuge tubes and centrifuged at 3000, r.p.m. for 10 minutes and clear straw-cloured fluid was dispense in 10ml amounts, inactivated in water-bath at 56°C for 10 minutes then stored frozen at -20°C until needed. This serum was used for serum agar.
2.3. Preparation of media:

2.3.1. Solid media:

2.3.1.1. Nutrient Agar:

Twenty-eight grams of dehydrated nutrient agar (Oxoid CM3) were suspended in 1 liter of distilled water, steamed to dissolve completely; the pH was adjusted to 7.4 and then sterilized by autoclaving at 121°C for 15 minutes.

2.3.1.2. Blood Agar Medium:

Forty grams of blood agar base (Oxoid CM55) were suspended in a liter of distilled water, steamed to dissolve, cooled and the pH adjusted to 7.4. It was then sterilized by autoclaving at 121°C for 15 minutes. After cooling to about 46°C, defibrinated sheep blood was added aseptically in 7-10% concentration.

The medium was then distributed in 20ml amounts in sterile Petri-dish.

2.3.1.3. Dorset egg medium:

The medium was prepared according to the method described by Cheesbrough (1984). The procedure was as follows: Two hen-eggs were cleaned and swabbed with 70% ethanol, dried, the shell cracked and the contents mixed thoroughly with 20ml of sterile nutrient broth to produce homogeneous mixture which was distributed in 2.5ml slow heating at 75°C in slope position and maintained at this temperature for one hour. The procedure was repeated for two consecutive days.

2.3.1.4. Serum agar medium:

The medium was prepared according to Cowan (1985) by addition of 10% sterile serum to melted nutrient agar.
2.3.1.5. Christensen's medium:

The medium was used for the detection of urea splitting organisms. It was prepared by dissolving 2.4 g of urea agar base (Oxoid) in 95ml distilled water, sterilized and cooled to 45°C before 5 ml of sterile 40% urea solution (Oxoid SR20) were added aseptically. The mixture was distributed in 10ml amounts into McCartney bottles and allowed to solidify as slopes.

2.3.1.6. Motility medium:

The medium was composed of tryptose, sodium chloride and agar. It was prepared according to manufacturer's instructions by dissolving 20g of the dehydrated powder in 1 liter distilled water, distributed in 5ml amounts into 10ml test tubes covered with cotton wool and sterilized.

2.3.2. Semi solid media:

2.3.2.1. Hugh and leifson's (O.F. medium):

The oxidation fermentation medium was used to test the ability of the organism to break down the carbohydrates by oxidation and/or fermentation. The medium contained peptone, sodium chloride, dipotassium hydrogen phosphate, agar and 0.2% aqueous solution of bromocresol purple. It was prepared according to Cowan (1985) by dissolving the solids in distilled water. The pH adjusted to 7.1, the medium was filtered and the indicator was added. Sterile solution of appropriate carbohydrate was then added aseptically to give a final concentration of 1%, the content was distributed aseptically in 10ml amounts into sterile test tubes and covered with cotton wool.

2.3.3. Liquid media:

2.3.3.1. Nutrient broth:

Thirteen grams of dehydrated (Oxoid, CM1) nutrient broth were suspended in a liter of distilled water, mixed well and the pH was adjusted to 7.4 and then sterilized by autoclaving at 121°C for 15 minutes.
This medium was used as base for carbohydrate utilization test and for other purposes.

2.3.3.2. Brain heart infusion broth:

This medium contained calf brain and beef heart infusion solids, peptone, dextrose, sodium chloride and di-sodium phosphate. It was prepared according to manufacturer's instructions by dissolving 37g of the powder in 1 liter distilled water and sterilized. 0.1 % of tween 80 was added to media before sterilized to enhance the growth.

2.3.3.4. Nitrate broth:

The medium was prepared according to Cowan (1985) by dissolving 1g of potassium nitrate in 1 liter of nutrient broth, distributed into bijou bottles in 3 ml amounts and sterilized.

2.3.3.5. Carbohydrate fermentation media:

An amount of 900ml peptone water was prepared and its pH was adjusted to 7.1, 10 ml of 0.2% aqueous solution of bromocresol purple were added and the medium then was sterilized. The sugar was prepared by dissolving 10 g in 90ml distilled water and sterilized. Sugar was added aseptically to sterile peptone water and the indicator. It was then distributed in 3 ml amounts into sterile bijou bottles with inverted Durham's tubes. All the bottles were labeled before use.

2.3.4. Reagents:

2.3.4.1. Tetramethyl-p-phenylene diamine dihydrochloride:

It was prepared fresh as 1% aqueous solution and used for oxidase test.

2.3.4.2. Hydrogen peroxide (H₂O₂):

It was prepared as 3% aqueous solution and used for catalase test.

2.3.4.3. Potassium hydroxide (KOH):

It was prepared as 40% solution and used in VP test.
2.3.4.4. Nitrate test reagent:

Nitrate reagent was composed of two solutions:
Solution (A): 0.8% sulphanilic acid in 5N-acetic acid. It was prepared by mixing 0.4ml of sulphanilic acid in 50ml of 5N-acetic acid. Solution (B): 0.5% dimethyl-α-naphthylamine in 5N acetic acid.

2.3.4.5. α-Naphthol solution:

It was prepare as 5% and used for VP test

2.3.5. Indicators:

2.3.5.1. Bromocresol purple:

It was prepared as 0.2% aqueous solution and used as indicator for different purpose.

2.3.5.2. Zinc powder:

It was used for nitrate reduction test in 5 mg/ml culture.

2.3.6. Solution:

2.3.6.1. Normal saline:

It was prepared by dissolving 8.5g sodium chloride in 1 liter distilled water.

2.4. Collection of samples:

Samples were collected from carcasses during meat inspection in Al Huda slaughterhouse at Omdurman Province. A total number of 157 grossly enlarged lymph nodes were collected, labeled, stored in ice box and transported to the Central Veterinary Research Laboratory.

2.5. Isolation of Corynebacterium pseudotuberculosis:

2.5.1. Primary isolation:

The fat and fascia covering the affected lymph nodes were removed using sterile scissors. A hot scalpel blade was singe the surface of the lymph nodes prior to incision aseptically, and swabs were taken from the periphery of the lesion and streaked into 7% sheep blood agar
plates. These were then incubated in a desicator, under 10% CO₂ tension at 37°C for 48 hours.

2.5.2. Purification

Typical and well isolated colony was purified by transferring to a blood agar plates and incubated aerobically at 37°C for 48 hour. This process was repeated till purification was obtained.

2.5.3. Identification of purified bacteria:

2.5.3.1. Cultural characteristic:

The growth characteristic of the colonies on blood agar plates including the colonial shape, size, colour and haemolysis were checked.

2.5.4. Primary test:

2.5.4.1. Gram's stain:

Smears were prepared by emulsifying a small inoculum in a drop of normal saline and spread it on a clean slide. The smears were allowed to dry in air and then fixed by gentle flaming. The prepared slides were placed on the rack and flooded with crystal violet stain for one minute. The slide was left covered with iodine for one minute, rinsed with water, acetone was poured for no time, and the slides were rinsed with water again. The slides were counter stained with carbol fuchsin for one minute. Rinsed in water and allowed it to dry by blotting it with filter paper.

2.5.4.2. Motility:

The test strain was inoculated into motility medium using straight wire and incubated at 37°C for 48 hours. Motile bacteria spread away from the inoculated line.

2.5.4.3. Aerobic and anaerobic growth:

Cultured media were incubated aerobically and anaerobically using Gas PAkJar.
2.5.4.4. Oxidase test:

The organism was taken by sterile glass rod and smeared in a surface of filter paper saturated by fresh 1% tetramethyl-p-phenylene diamine dihydrochloride. Positive reaction indicated by formation of purple color within 10 seconds.

2.5.4.5. Catalase test:

The organisms were streaked on serum agar, incubated at 37°C for 48 hours. A small amount of culture was picked with sterile Pasteur pipette, placed on clean slide, and then a drop of 3% aqueous solution of hydrogen peroxide was placed over the culture and observed for production of gas bubbles during 10 seconds.

2.5.4.6. Oxidation fermentation of carbohydrate:

Two tubes containing Hugh and Leifson's medium were inoculated with the test organism by stabbing with straight wire. The medium in one of the inoculated tubes was covered with a layer of sterile liquid paraffin oil to depth of 1 cm. The tubes were incubated and examined daily for 14 days, change of colour to yellow in both tubes indicated that the organism is fermentative, but the yellow colour in the open tube only indicated that the organism is oxidative.

2.5.5. Biochemical test:

2.5.5.1. Sugar fermentation:

Each test organism was cultured in Bijou bottles containing various peptone water sugars, incubated at 37°C and examined for acid and gas production during 14 days. Positive reaction was indicated by changes of colour to yellow due to acid production, and Durham's tubes examined the gas production. This test was carried out on media containing glucose, maltose, and lactose.
2.5.5.2. Nitrate reduction:

Inoculated nitrate broth was incubated for up to five days. Then one ml of nitrate reagent solution (A) was added followed by 1 ml of nitrate reagent solution (B).

Positive reaction was indicated by the development of red colour. To tube not showing red colour within five minutes, powdered zinc was added, the development of red colour indicated that nitrate was not reduced.

2.5.5.3. Urease test:

A urea agar slant was streaked with the test organism and incubated at 37°C for 24-48 hours. The development of a pink colour was indicative of production of NH₃. Negative and weak tests were left for a week before taking results.

2.5.5.4. Voges-Proskauer test:

Glucose phosphate broth was cultured with the test culture and incubated for 48 hours. To five ml of culture, 0.6ml of 5% α-naphthol was added followed by 0.2ml of 40% KOH. The tube was thoroughly mixed and sloped for up to 60 minutes. Positive reaction was indicated by a strong red colour.

2.5.6. Storage and labeling of bacterial isolates:

The isolates which were considered to be Corynebacterium were carefully labeled and preserved on blood agar slant, Dorset egg medium and lyophilized (stored in -20°C).

2.5.6. Inhibition of staphylococcal beta toxin:

This test was done according to the method described by Lovell and Zaki (1966). In this test Corynebacterium pseudotuberculosis exotoxin occupied the receptor site on the erythrocytes membrane preventing the beta lysin from exerting its haemolytic effect. This method had sensitivity and specificity level of 92%-96% respectively.
Four colonies of *Corynebacterium pseudotuberculosis* were cultured in a four corners of proposition square had equal diameter on bovine blood agar plate and in the middle of the square one colony of *Staphylococcus aureus* was cultured.

2.6. Determination of short chain fatty acids composition by Gas liquid chromatography:

2.6.1. Sample preparation for GLC analysis:

Direct transmethylation of fatty acids from examined bacterial samples without prior exteraction was done as described by (Dionisi *et al.*1999).

One ml of Methanolic HCl (1.5 M) was added to 100mg of dried bacterial samples, vortexed for 5-10 sec and 1 ml of methanol was also added. The sample was incubated in a water bath at 80°C for 10 min. Two ml of distill water were added and finally centrifuged at 2500rpm for 5 min. The upper two third of the supernatant was used for gas liquid chromatography analysis.

2.6.2. Preparation of standard:

The standards acetic acid, propionic acid, butyric acid, valeric acid, isovalaric acid, caporic acid and isocaporic acid were prepared as described by Holdman and Moore (1975).

A 0.4 ml of 50% H₂SO₄ and 0.8g NaCl were added to 2 ml of standard. Two ml of diethyl ether was added, the tube was stoppered and shaked about 20 times. Centrifugation at 2500 rpm for 5 min was done to break the emulsion and the ether layer was pipetted off.

2.6.3. Gas liquid chromatography:

Samples were analyzed in gas chromatograph (Shimadzu GC Model 2010). Routine condition of analysis were: detector (Flame
ionization detector) 110°C, injector pore 110°C, column (DBI 30 meter) 95°C. The carrier gas was nitrogen. The sample size was 2µl.

2.7. Identification of *Corynebacterium pseudotuberculosis* isolated from sheep by PCR:

2.7.1. DNA extraction:

A 10 ml of fresh brain heart infusion broth culture was centrifuged at 4C (2000g for 20mi) and chromosomal DNA extraction was performed by Quiageen kits.

2.7.2. Primer and PCR condition:

The primer used in this study is targeting the 16rRNA gene of *Corynebacterium pseudotuberculosis* was obtained from previously published work (Cetinkaya *et al.* 2002).

The length of the primers were 20bp and the annealing temperature was 55°C. The forward primer had the sequence ACCGCACTTTAGTGTGTGTG (*E. coli* position 183-203) and the reversed primer was TCTC TACGCCGATCTTGTAT (*E. coli* position 1019-999).

The PCR was performed in a thermocycler (PeQlab biotechnologie GmbH, Germany) in a final reaction volume of 25µl containing 2.5 µl of PCR Buffer, MgCl 50UM (1 µl), 1 µl of each deoxynucleotide triphosphate, 0.4µl of Tag DNA polymerase, 1µl of each primer, 2.5µl of template DNA and 15.6µl D.W. Amplification was obtained with 30 cycles following initial denaturating step at 94°C for 5 min. Each cycle involved denaturation at 94°C for 1 min., annealing at 56°C for 1 min., and synthesis at 72°C for 2 min.

2.7.3. Electrophoresis:

The amplified products were detected by ethidium bromide staining after electrophoresis for 1 hour in 1.5% agarose gel.
2.8. Pathogenicity in mice:

This experiment was done by using five strains according to the differences in the biochemical activities and fatty acids contents. One colony of each strain from blood agar plates was inoculated in 5ml of sterile brain heart infusion broth plus (0.1%) Tween 80 and incubated for 48 hours at 37°C. A dose of 0.2 ml of fresh culture was injected s/c to a group of five mice each. Control group was injected s/c by 0.2 ml of sterile normal saline.

Mice were observed for 10 days post inoculation for deaths and abscess development. Dead as well as surviving animals were autopsied and their internal organs (lung, heart, liver, spleen, kidney) were inspected, smeared and Gram stained, and cultured on blood agar plates. A small part of these organs were fixed in 10% formalin and processed for histopathology.


Chapter three
Results

3.1. Post mortem findings:

A total numbers of 3875 sheep carcasses were examined and a total of 157 lymph node abscesses were collected for bacteriological examinations.

From 129 infected sheep carcasses 104 had a single lymph node abscess and in 25 sheep carcasses the infected lymph nodes were found in two or more different sites at the same carcasses.

Table (1) showing the general features of abscessation in investigated sheep.

Table (2) showing the distribution of lesions in inspected sheep carcasses.

From 157 lesions colleted in this study, *Corynebacterium pseudotuberculosis* isolated from 49 samples.

3.2. Microbiological and biochemical examinations:

3.2.1. Morphology and staining:

In Gram-stained smears the test organism appeared as Gram-positive, the cell range from coccoid to filamentous, exhibiting a tendency to form clumps palisade arrangement simulating Chinese letters. The organism was non motile, non sporforming and unencapsulated.

3.2.2. Growth characteristics

3.2.2.1. Blood agar medium:

The organism was cultured in blood agar medium and incubated at 37°C for 48 hours. Colonies were off-white, dull, faintly haemolytic and about 1mm in diameter. The colonies could be pushed across the agar medium without disintegrating (some describe this as pushing a "hockey puck").
3.2.2.2. Serum agar:
Colonies were small, translucent and as colonies aged they became opaque, dry and crumbly with cream to orange colour.

3.2.2.3. Dorset egg medium
On the surface of the slope the organism was forming a thin yellowish film after 48 hours of incubation at 37°C.

3.2.2.4. Brain heart infusion broth:
In this medium the growth obtained after 48 hours forming granular deposit with a surface pellicle with no general turbidity.

3.2.3. Biochemical properties:
All isolates of *Corynebacterium pseudotuberculosis* were negative for oxidase, lactose, V.P. and did not reduce nitrate. But all isolates were positive to catalase, urease and starch. All isolates produced acid but not gas from fermentable sugars. Table (3) showing the biochemical characteristics of *C. pseudotuberculosis* isolated from sheep carcasses.

3.3. Inhibition of staphylococcal beta toxin:
All strains tested were inhibited staphylococcal beta toxin (phospholipase c) activity on bovine blood agar by *C. pseudotuberculosis* (phospholipase D).

3.4. Gas liquid chromatography:
In all strains tested by GLC Propionic acid was found and formed the highest peak but Valaric acid was not detected.

The percentage composition of the short chain fatty acid from 20 strains examined shown in fig 3,4,5,6 and table (3).

3.5. Identification of *Corynebacterium pseudotuberculosis* by PCR:
PCR products with the molecular size of 815 bp were considered as *Corynebacterium pseudotuberculosis*. The five strains were positive by PCR.
3.6. Pathogenicity results:

The five strains different in their pathogenicity to mice. Strains 2 and 13 were the most pathogenic, caused acute disease and death during 2-5 days. The organism was demonstrated in Gram-stained smears and re-isolated from the liver, spleen, kidney and lung.

Strains 11 and 65 was less pathogenic, caused death during 4-7 days, the mortality rate was 80% and 60% respectively and the organism were isolated from the internal organs on blood agar plates.

Strain 64 was not pathogenic but the organism was isolated from internal organs.

3.6.1. Post mortem findings:

The internal organs was congested and the spleen was enlarged and congested.

3.6.2. Histopathological findings:

Histopathological changes in all groups was same but the differences was in the severity of changes.

In the liver there was haemorrhage, congestion, focal inflammatory cells and hepatic cells showed necrosis with pyknotic nuclei and cytoplasmic vacules. The kidney showed congestion, haemorrhage, and renal tubules necrosis.

In the lung there was congestion and haemorrhage, thickening of alveolar septa and inflammatory cells were also observed. In the heart there was cardiac muscle degeneration.

The spleen showed depletion of lymphocytes, giant cells and RBCs.
**Table 1: The distribution of abscesses in inspected sheep carcasses.**

<table>
<thead>
<tr>
<th>Description</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of sheep examined</td>
<td>3875</td>
<td></td>
</tr>
<tr>
<td>Infected sheep</td>
<td>129</td>
<td>3.32%</td>
</tr>
<tr>
<td>Carcasses with single lesion</td>
<td>104</td>
<td>80.62%</td>
</tr>
<tr>
<td>Carcasses with two lesions</td>
<td>23</td>
<td>17.83%</td>
</tr>
<tr>
<td>Carcasses with three lesions</td>
<td>2</td>
<td>1.5%</td>
</tr>
</tbody>
</table>
Table 2: Distribution of lesions in examined sheep carcasses

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lesions</td>
<td>157</td>
</tr>
<tr>
<td>Mandibular</td>
<td>9 (5.78)</td>
</tr>
<tr>
<td>Parotid</td>
<td>41 (26.11)</td>
</tr>
<tr>
<td>Prescpular</td>
<td>30 (19.10)</td>
</tr>
<tr>
<td>Precrural</td>
<td>22 (14.01)</td>
</tr>
<tr>
<td>Popliteal</td>
<td>15 (9.55)</td>
</tr>
<tr>
<td>Inguinal</td>
<td>8 (5.09)</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>12 (7.64)</td>
</tr>
<tr>
<td>Hepatic</td>
<td>14 (8.91)</td>
</tr>
<tr>
<td>Mediastinal</td>
<td>6 (3.81)</td>
</tr>
</tbody>
</table>
Table 3: Biochemical characteristics of *Corynebacterium pseudotuberculosis* isolated from abscesses.

<table>
<thead>
<tr>
<th>Test</th>
<th>Reaction (%)</th>
</tr>
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<tbody>
<tr>
<td>Oxidase</td>
<td>-ve (0%)</td>
</tr>
<tr>
<td>Catalase</td>
<td>+ve (100%)</td>
</tr>
<tr>
<td>O.F.</td>
<td>+ve (100%)</td>
</tr>
<tr>
<td>Glucose</td>
<td>+ve (100%)</td>
</tr>
<tr>
<td>Lactose</td>
<td>-ve (0%)</td>
</tr>
<tr>
<td>Maltose</td>
<td>+ve (93.8%)</td>
</tr>
<tr>
<td>Urease</td>
<td>+ve (100%)</td>
</tr>
<tr>
<td>V.P.</td>
<td>-ve (0%)</td>
</tr>
<tr>
<td>Nitrate</td>
<td>-ve (0%)</td>
</tr>
</tbody>
</table>
Fig. (1): Gram stained *Corynebacterium pseudotuberculosis*.

Fig. (2): Inhibition of Staphylococcal beta toxin activity on bovine blood agar by *Corynebacterium pseudotuberculosis* (phospholipase D).
Fig. (3): % composition of short chain fatty acid of *Corynebacterium pseudotuberculosis* isolates.

Fig. (4): % composition of short chain fatty acid of *Corynebacterium pseudotuberculosis* isolates.
Fig. (5): % composition of short chain fatty acid of *Corynebacterium pseudotuberculosis* isolates.

Fig. (6): % composition of short chain fatty acid of *Corynebacterium pseudotuberculosis* isolates.
Fig 7: Identification of *Corynebacterium pseudotuberculosis* isolates by PCR.
Fig. (8): Stain H&E (X10). Congestion, haemorrhage and granuloma in kidney.

Fig.(9): Stain H&E (X40). Focal inflammatory cells and congestion in liver.
Fig. (10): Stain H&E (X40). Congestion, thickening in alveolar septa and inflammatory cells in lung.

Fig. (11): Stain H&E (X40). Haemorrhage and cardiac muscle degeneration in heart.
Fig. (12): Stain H&E (X40). Depletion of lymphocytes, Giant cells and RBCs in spleen.
Chapter Four
Discussion

Caseous lymphadenitis of sheep has been a significant disease in the majority of sheep-rearing regions for over a century, because of the chronic and often subclinical nature of the infection.

In Sudan the disease is associated with economic losses in a variety of ways, which include meat condemnation and down grading of hide. Labour cost and antibiotics used in combating the disease were additional factors of economical loss.

The purpose of this study was isolation of *corynebacterium pseudotuberculosis*, compare the percentage composition of short chain fatty acids from isolated strains, identification of isolates by PCR and study their pathogenicity in mice.

In Khartoum state, Khalid (1971) studies the causative organisms isolated from caseated lymph nodes of sheep while Hamad *et al.* (1992) dealt with the aetiology of abscesses of sheep and Ahmed (1994) investigated the sources of infection. Elgaddal (1997) studied the prevalence, aetiology and diagnosis of CLA by serological tests in South Darfur State.

In this study the lesions of sheep abscesses were found in order of frequency of infection in the parotid, prescapular, precrural ad popliteal lymph nodes. The parotid lymph nodes were found to be the most affected ones. Our findings are in agreement with (Asfag and Campell, 1979) and Elgaddal (1997) who suggested that ear marking which takes the form of certain cuts for identification might be a possible route of infection.
Carne (1939), Maddy (1953) and Batey (1986) suggested that infection by *C. pseudotuberculosis* occurred through wounds or mucus membranes followed by spread to the regional lymph nodes. This suggestions supported the findings reported in this study.

From 157 pus samples, 6 samples were bacteriological negative. These findings were in agreement with these of Aynaud (1923) and Carre (1923b) who reported that only one pus sample out of 37 and 20 respectively were bacteriologically negative. Also Elgaddal reported that 15 out of 351 (4.27%) of pus samples were bacteriologically negative. This could be attributed to the destruction of bacteria by host defense mechanisms.

Kuria and Ngatia (1990) stated that the pus was green-yellow to creamy greenish-yellow, where a Ayers (1977), Stoops (1984) and Lioy *et al* (1990) described the pus as thick and greenish white in colour. However, the degree of green colouration was due to the presence of the enzyme myeloperoxidase (Tizard, 1992).

From our observations during this study the lesions of CLA containing cheesy pus. This was in agreement with the finding of Hamacd *et al* (1992) and Elgaddal (1997).

Identification of *C. pseudotuberculosis* by specific-PCR might be useful for several reasons. First, the antigenic similarities between *Corynebacterium* species is the major cause of a significant number of false-negative and false-positive results in serological test. This has hindered the use of serological test to monitor C.L.. Second, bacteriological test have some drawbacks as well. The most important drawbacks is to variation in biochemical characteristics of *Corynebacterium* species which has hampered the use of routine bacteriology in differentiating the species belonging to the genus (Hommez *et al*., 1999).
In this study *C. pseudotuberculosis* specific PCR was used to identify 5 strains which had difference percentage composition of short chain fatty acid and used in pathogenicity experiment.

In this study we used gas liquid chromatography for qualitatively and quantitatively assess of short chain fatty acid among 20 isolates.

Propionic acid formed the highest peak, butric, isovalaric, caporic and isocaporic acid are also present but none of them as major peak. These finding agreed with Reddy (1978) who find that *C. psudotuberculosis* produced a major amount of propionic acid and formic acid as a metabolic products.

The highly pathogenic strains have a high content of propionic acid while the non pathogenic strain has low concentration of propionic acid.

In view of that, we put forward, the fact that the content of propionic acid might has a direct influence on the pathogenicity of *C. pseudotuberculosis*.

From the results of pathogenicity experiment it was shown that *C. pseudotuberculosis*, isolated from a natural case of caseous lymphadenitis could cause acute disease in white mice. Other aspect that must be considered is the period of observation was not long enough for abscesses formation in the internal organs and site of inoculation.

The important finding was the intense infiltration of polymorphoneuclear inflammatory cells and that indicating acute infectious process. Similar findings were observed by Junior and Oliveria (2006) in their study of experimental infection of goats by *Corynebacterium pseudotuberculosis*. 
Conclusions and Recommendations

Conclusions:
From the results obtained we can conclude the following:

- There were qualitative and quantitative differences in short chain fatty acids among the isolates and this is a considerable taxonomic value to differentiate between strains.

- The local Sudanese isolates of *C. Pseudotuberculosis* are genetically identical.

Recommendations:

- Accordingly, more work is warranted to study more other factors and mechanisms of virulence.

- From my opinion, accurate serodiagnosis together with vaccination must be the main strategy to control CLA in Sudan.
References


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