PATHOGENESIS AND IMMUNE RESPONSE TO CASEOUS LYMPHADENITIS IN GOATS.

BY

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DECLARATION.

This thesis is my original work and has not been submitted for a degree award in any other University.

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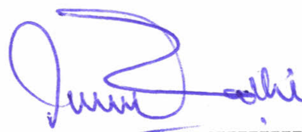

Dedication.

This thesis is dedicated to my daughter "**Tuti**" whose boundless energy was a constant challenge.

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SUMMARY.

Effective immunisation against caseous lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis*, in sheep and goats is hindered by inadequate information on the pathogenesis of the disease and the immune response against the organism. An investigation into the pathogenesis and immune response to the causative agent in goats was studied. The pathogenesis was investigated by :- examination of natural CLA lesions in sheep and goats, experimental infection of goats through different routes, determination of the minimum dose of infection for development of CLA lesions, determination of the incubation period for development of CLA lesions, and determination of the ability of two factors, (the surface lipid and the extracellular toxin), and three whole- cell preparations of *C. pseudotuberculosis* to produce CLA lesions. The immune response was investigated by serological analysis of sera from both naturally and experimentally infected animals using bacterial agglutination test (BAT) and the haemolysis inhibition test (HIT).

CLA lesions in goats and sheep in Kenya were found to be mainly in prescapular followed by precrural lymphnodes. Lesions were also observed in parotid and submaxillary lymphnodes, the lungs and subcutaneous tissue. The prevalence of CLA in Kenya was found to be 7.1% and 1.8% in goats and

sheep respectively. *C. pseudotuberculosis* was isolated from 79.4% of lesions. Other microorganisms either in pure or mixed cultures (*Bacillus* spp, *Streptococcus* spp, *Staphylococcus* spp, coliforms and *Pseudomonas* spp) were isolated from 11.1% while 9.5% of lesions were found sterile.

Grossly, CLA lesions were either caseous, granular or in some cases calcified. Microscopically, the lesions were those of typical capsulated granulomatous reactions. Evidence from early lesions indicated that the necrotic centre was composed mainly of dead mononuclear cells. Evidence of resolution of lesions by formation of granulation tissue was also observed.

In serological analysis, the BAT and HIT tests were found to detect 87.7% and 80.7% respectively of the infected animals while the combined tests could detect 89.5%. The specificity of the tests was calculated as 88% for BAT, 97.2% for HIT and 87.0% for the combined tests.

Investigation into the routes of infection indicated that CLA could be induced by subcutaneous or intradermal injection of caseous abscess material or by smearing the caseous pus on scarified skin but not on intact shaved skin. Haematological findings indicated that a leucocytosis, characterised by a neutrophilia, occurred during the first three weeks post infection but no significant changes occurred in other parameters (PCV, Hb and total plasma protein concentration). Positive BAT and HIT titres could be detected two weeks post infection. Antibody response was dependent on the route of infection, with a decrease from intradermally infected to subcutaneously infected to scarified skin infected.

The minimum dose of *C. pseudotuberculosis* for induction of CLA lesions through intradermal route was 10^2 colony forming units (cfu), per lymphnode. Doses

of 5 x 10^1 cfu did not induce CLA lesions. Serological analysis results indicated that antibody response decreased with decrease in dose of infection. Animals infected with doses equal to or less than 10^3 cfu and doses of 10^2 cfu did not develop positive BAT and HIT titres respectively although they developed CLA lesions. This was an indication that low doses of *C. pseudotuberculosis* may induce CLA lesions without eliciting a detectable antibody response.

The incubation period for development of CLA lesions was between 8-9 days after intradermal infection with caseous pus material or after infection with caseous pus on scarified skin. Microscopic lesions could, however, be detected as early as two (2) days post intradermal infection. This indicated that although CLA is chronic in duration, it is subacute in development. BAT and HIT titres were recorded from 15-17 days and 20-25 days respectively, post infection. This was an indication that serology may be of no value in detection of CLA during the period 2-3 weeks after infection.

Animals injected intradermally with the waxy lipid or extracelluar toxin preparations developed abscesses at injection sites as well as granulomatous lesions in draining regional lymphnodes. The lymphnode lesions were similar to the early-stage lesions produced by the live organism. The heat-treated toxin produced no lesions. Animals injected with the heat-killed, formalin-killed or ether/ethanol extracted cells developed abscesses at the injection sites only. It was concluded that both the waxy lipid and the extracellar toxin play a major role in development of caseous lymphnode lesions. Antibodies were detected in animals injected with the

potent toxin, heat- killed and formalin killed cells and with ether/ethanol extracted cells but not in animals injected with either the waxy lipid or heat-treated toxin. The best antibody response was elicited by the ether/ethanol extracted cells. This was an indication that ether/ethanol extraction preserved antigenicity of *C. pseudotuberculosis* better than either formalin or heat treatment.

1. INTRODUCTION.

Caseous lymphadenitis (CLA) is a disease of world wide importance in sheep and goats. It is caused by the intracellular bacterial parasite, *Corynebacteium pseudotuberculosis*. The disease is characterised by suppurative lymphadenitis especially of the peripheral lymphnodes (Addo and Eid, 1978; Jubb *et al.*, 1985). Occasionally the condition becomes generalised to involve internal lymphnodes and visceral organs (Renshaw *et al.*, 1979; Jubb *et al.*, 1985,). When a large number of lymphnodes are affected or when disemination into visceral organs has occured, varying degrees of debilitation are common (Addo and Eid, 1978). This chronic debilitating condition is indistinguishable from other chronic diseases. The resultant economic losses are due to decreased production and reproduction as well as condemnation of carcasses during slaughter (Renshaw *et al.*, 1979; Hein and Cargill, 1981; Batey, 1986a).

Control of CLA has been a major concern in the sheep and goat industry. Once introduced into a herd or farm, the disease is almost impossible to eradicate. The organism can survive for several weeks on common barnyard fomites especially in the presence of organic matter (Augustine and Renshaw, 1982a; 1982b; 1986). Chemotherapeutic treatment of affected animals is not effective due to the poor penetration of antimicrobial drugs into the usually capsulated lesions (Ashfaq and Campbell, 1979). Effective control of CLA should therefore be approached through preventive immunisation. Athough regular cleaning and disinfection of housing can remove the organism, contamination by ruptured abscesses soon occurs.

Attempts to immunise animals by vaccination have however been largely unsuccessful (Cameron *et al.*, 1972; Abdel-Hamid and Zaki, 1973; Cameron and Bester, 1984; Brogden *et al.*, 1984; Brown *et al.*, 1986a; Holstad *et al.*, 1989). Although vaccinated animals tend to resist the lethal subacute disease after challenge, they are unable to resist the abscess -

forming chronic condition that is characteristic of CLA. A vaccine developed for sheep by Commonwealth Scientific and Industrial Research Organisation (CSIRO) laboratories in Australia, "Glenvac", has been found to have low efficacy (Baxendel, 1984). More research into the pathogenesis of CLA and the hosts immune response to the organism would therefore be necessary. This information is essential in development of an effective control programme for CLA.

The current status of CLA in Kenya is not known. Shirlaw and Ashford (1962), observed lymphnode swellings in an exotic sheep flock and reported isolation of a *Micrococcus* organism. Small ruminants in the country are reared mainly in open rangelands. With increasing pressure on land and also the necessity for conservation, production is expected to be more and more intensive. CLA is a major problem where intensive husbandry of small ruminants is practised (Gilmour, 1990).

CLA primarily affects the superficial lymphnodes but animals can have internal undetectable lesions (Hein and Cargill, 1981; Anderson and Nairn, 1985; Batey *et al.*, 1986). The morphological appearance of the lesions is different in sheep and goats (Ayers, 1977). In sheep, the abscesses have a characteristic lamellated appearance and often calcified while in goats the exudate is usually uniformly pasty and rarely calcified (Brown and Olander, 1987). The aetiological agent *C. pseudotuberculosis* is not always recovered from the lesions. Other microoganisms, thought to be secondary invaders or contaminants, are sometimes isolated (Renshaw *et al.*, 1979). Similarly, antibodies to *C. pseudotuberculosis* are not always detectable in affected animals and *vice versa* (Brown *et al.*, 1986b). Current information on the relationship between the presence of CLA lesions, the presence of the infectious organism in the lesions and the antibody status of the animals is inadequate. The use of serology in identification of infected individuals need to be evaluated.

Infection by *C. pseudotuberculosis* takes place through skin wounds such as those inflicted during shearing in sheep and by thorns, barbed wire and other sharp objects in goats (Nairn *et al.*, 1977; Addo and Eid, 1978; Hein and Cargil 1981 Brogden *et al.*, 1984). The source of infection is pus from discharging ruptured lymphnodes or subcutaneous abscesses, which directly contaminate wounds on other animals or indirectly through contamination of enviroment (Augustine *et al.*, 1982).

The assessment of vaccination as a means of control is hindered by inconsistency in experimental disease induction/challenge systems. The cause of the inconsistency appears to be the choice of route and dose of infection. There is also no information on relationship between the route of infection and antibody response.

The reported dosage of *C. pseudotuberculosis* sufficient to cause abscessation of regional lymphnodes varies widely. Abdel- Hamid and Zaki (1972), used 0.01- 0.25 mls of a 48 hour broth culture ; Holstad and Teige (1988b), and Ashfaq and Campbell (1979), used 1 x 10^6 colony forming units (cfu); Maki *et al.*, (1985) reported a minimum dose of 1 x 10^4 ; and Pepin *et al.*, (1988) used a dose of 1 x 10^8 cfu. The dose may be influenced by the route of infection and the pathogenicity of the strain used. Information on the dose of infection for CLA is essential in understanding the natural transmission of the disease and also in establishing immunisation and or challenge systems. Information on the relationship between the dose of infection and the immune response is also lacking. This would give an indication as to whether or not some dosages can cause lesions without eliciting antibody response and *vice-versa*.

Caseous lymphadenitis is described as a chronic disease. There is however no clear information on the incubation period for development of lesions. There is also no information on the pathogenesis of the lesions in the lymphnodes, leading to the formation of gross

lesions. The incubation period is an important epizootiological factor. It gives an indication of how soon the disease can be detected after exposure and also how soon an infected animal can transmit the disease. Cellular changes in lymphnodes are an indication of the host's cellular defence mechanism. The relationship between the incubation period and antibody responce has not been investigated. This would give information on the usefulness of serodiagnostic tests, at various stages of infection.

The pathogenic components of *C. pseudotuberculosis* are believed to be the surface waxy lipid and the extracellular toxin. The lipid contains corynomycolic acids similar to the mycolic acids of Mycobacteria (Barksdale, 1981). The substance protects the organism from intracellular degradative enzymes, thus allowing the organism to survive intracellularly within phagocytes (Hard, 1972). The lipid has also been considered to be the pyogenic factor responsible for formation of abscesses through leucocyte cytotoxicity (Carne *et al.*, 1956; Hard, 1975; Muckle and Gyles, 1983; Tashjian and Campbell, 1983). There is no information on whether or not the substance can produce lymphnode abscesses. There has been no investigation into the role of the organism's extracellular toxin in the formation of CLA lesions, although it is thought to contribute through intracellular cytotoxicity (Burrel, 1978a). The toxin, a phospholipase D, is thought to act as a permeability factor (Jolly, 1965a; Carne and Onon, 1978). The site of action of the enzyme is the sphingomyelin component of the capillary cell membrane (Bernheimer *et al.*, 1980).

The objectives of the study were:-

1. Determine the prevalence and the pathological manifestation of CLA in goats and

sheep, the morphological and the bacteriological content of the lesions, the antibody status of affected animals and the usefullness of serological tests in detection of naturally infected animals.

2. Investigate the possible routes of infection for CLA and the resultant clinical and serological changes.

3. Determine the minimum dose of *C. pseudotuberculosis* necessary to establish CLA lesions in draining lympnodes and the relationship between the dose of infection and antibody response.

4. Determine the incubation period for the development of CLA lesions in draining lympnodes and the relationship between the incubation period and the clinical and serological changes.

5. Examine the ability of the surface lipid and the extracellular toxin of *C. pseudotuberculosis* to produce lympnode lesions and the serological response to the substances.

2.0. REVIEW OF LITERATURE.

2.1. The organism.

2.1.1. History and Nomenclature.

The organism was first isolated in 1884 by a French veterinarian, Edmond Nocard, from a case of bovine lymphangitis (Benham *et al.*, 1962). A similar bacterium was isolated three years later by Hugo Van Preisz in Budapest from a renal abscess in a sheep (Collins and Cumming, 1086). Subsequently the organism became known as the Preisz-Nocard bacillus. Preisz later, (1888) described the organism in detail and named it *Bacillus pseudotuberculosis ovis* (Collins and Cumming, 1986). Buchanan, (1911) simplified the name to *Bacillus pseudotuberculosis* by dropping the subspecies designation and Eberson (1918) renamed it *Corynebacterium pseudotuberculosis* after it was noted that the organism had a distinctly diphtheroid morphology (Benham *et al.*, 1962; Collins and cummings, 1986). In the Bergey's manual of 1923, the organism was named *Corynebacterium ovis* but on realization that the organism had a wide host range, it was officially named *Corynebacterium pseudotuberculosis* in 1948. However, the name *C. ovis* is still used as a synonym (Collins and Cumming 1986). The species designation "pseudotuberculosis" is an attribute to the lesions, "false tubercles"' produced by the organism, which resemble those of tuberculosis.

2.1.2. Morphology and staining

The morphology of the bacterium has been adequately reviewed by Benham *et al.*, (1962) and Brown and Olander, (1987). The organism, together with other members of the genus *Corynebacteria,* is referred to as a diphtheroid due to the beaded, club-shaped appearance. It is a non-motile, non-spore forming, straight to slightly curved, very short Gram-positive rod. Often, it has club-shaped ends (Hard, 1969a). It measures 1-3μm by

0.5-0.6 μm (Brown and Olander, 1987). It is highly pleomorphic and varies from coccoid to bacillary form in natural lesion to coccobacillus in culture (Carne, 1939; Nadim and Farid, 1973; Brown and Olander, 1987). Branching forms may be observed in old cultures (Carne, 1939; Nadim and Farid, 1973). The large forms contain irregularly- staining granules referred to as metachromatic granules (Carne, 1939; Purchase, 1944; Brown and Olander, 1987). The organism is non-acid fast (Carne, 1939; Purchase, 1944). It undergoes snapping binary fission in which the daughter cells produce a palisade or "chinese letter" arrangement.

The organism contains large amounts of a waxy lipid external to the cell wall (Carne *et al.*, 1956; Jolly, 1966; Hard 1969a) which may be several times thicker than the actual organism (Hard, 1969a) and may account for as much as 11.3% dry weight of the bacterial cell (Ioneda and Silva, 1979). The waxy lipid consists of mycolic acids, referred to as corynomycolic acids, similar to those found in *Mycobacterium* species (Lancave *et al.*, 1967). The waxy lipid is responsible for the spontaneous autoagglutination of the organism in suspension (Keskintepe, 1976a).

The autoagglutination can be inhibited by detergents such as Tween 80 (Keskintepe, 1976a) and the waxy lipid is extractable with organic solvents such as ether/ethanol or petroleum spirit, without eliminating the viability of bacterial cells (Carne *et al.*, 1956).

2.1.3. Cultural characteristics.

The organism is a facultative anaerobe that grows slowly. After 24 hours, colonies on solid media are pinpoint greyish-white. After 48 hours they are greyish-white, umbonate,about one mm in diameter and with a smooth shiny surface which on further incubation becomes matt. On blood agar, colonies are yellowish and are surrounded by a narrow zone of beta-haemolysis. Further incubation produces another

wider zone of partial haemolysis especially on incubation at 4°C (Zaki, 1965a; Lovell and Zaki, 1966). Colonies have a waxy or butyrous consistency, due to the high lipid content and are not easily emulsifiable (Nadim and Farid, 1973). In broth, *C. pseudotuberculosis* grows as a pellicle due to the hydrophobic nature of the cell surface (Carne, 1939). Strains with less amounts of lipid are less hydrophobic and have less tendency to form pellicles (Jolly, 1966; Hard 1969a). Less hydrophobic colonies can be obtained by repeated subculturing (Hard, 1969a Barakat *et al.*, 1970).

2.1.4. Biochemical characteristics.

C. pseudotuberculosis forms acid from a number of carbohydrates viz. glucose galactose, maltose and mannose (Barksdale, 1981; Muckle and Gyles, 1982). Carne, (1939) and Nadim and Farid, (1973) found all isolates from cattle and sheep to ferment glucose, fructose and glycerol. The organism is strongly catalase positive. Most strains produce urease (Keskintepe, 1976b; Songer *et al.*, 1988) but attenuation by repeated subculturing has been found to produce urease negative strains (Barakat *et al.*, 1970; Burrell, 1979).

Nitrate reduction is variable. Strains isolated from sheep and goats tend not to reduce nitrates to nitrites whereas those isolated from horses invariably do so (Brown and Olander, 1987). Biberstein *et al.*, (1971) found bovine isolates to reduce nitrates. *C. pseudotuberculosis* strains can therefore be separated into two biotypes on this basis i.e a nitrate-reducing biotype that infects mainly horses and a nitrate negative biotype that infects mainly cattle, sheep and goats (Biberstein *et al.*, 1971; Barakat *et al.*, 1984; Songer *et al.*, 1988). The difference between the two biotypes is correlated with serological difference in gel precipitation reaction using sodium desoxycholate - extracted antigen (Barakat *et al.*, 1984) and by results of restriction endonuclease analyses (Songer *et al.*, 1988).

2.1.5. Metabolic products.

C. pseudotuberculosis produces elaborates an exotoxin detectable in cell-free culture supernatant (Carne *et al.*, 1956). The exotoxin is a phospholipase D (Soucek *et al.* 1967; 1971) and functions as a sphingomyelinase, splitting sphingomyelins, an important cell membrane component into ceramide phosphate and choline (Soucek and Souckova, 1974; Carne and Onon, 1978; Bernheimer *et al.*, 1980). The exotoxin has similar activity to the toxic enzyme found in the brown recluse spider, *Loxosceles reclusa* (Bernheimer *et al.*, 1985). The exotoxin has been characterised as a glycoprotein with an amino acid sequence similar to that of collagen (Onon, 1979). It has a molecular weight of about 31,000 daltons (Hsu, 1984; Bernheimer *et al.*, 1985). It is found in the cytoplasm and in smaller amounts in the cell wall. In culture, maximum production of exotoxin occurs at 30°C (Doty *et al.*, 1964). It is inactivated by heat, acid pH (less than 5) and formalin (Carne, 1940; Zaki 1965a). Phospholipase D splits membrane sphingomyelin of erythrocytes to cause partial haemolysis (Zaki 1965a). Chilling or decreasing the pH to below 6 will convert the partial haemolysis to frank haemolysis (Burrell, 1979).The exotoxin of *C. pseudotuberculosis* interacts with metabolites from other organisms to produce varying biological phenomena on animal erythrocytes. It inhibits haemolysis by the ß-haemolysin of *Staphylococcous aureus,* also a sphingomyelinase, by competitively occupying the target site on the erythrocyte membrane (Zaki, 1965b; Lovell and Zaki, 1966; Linder and Berheimer, 1978). This phenonenon is the basis for the antihaemolysin inhibition test (AHI) for detection of antibodies to *C. pseudotuberculosis* exotoxin (Zaki, 1968). *C. pseudotuberculosis* phospholipase D acts in concert with phospholipase C of *Rhodococcus equi* to produce synergistic haemolysis. The phospholipase C of *Rhodococcus equi* further degrades the ceramide phosphate produced from cleavage of sphingomyelins by phospholipase D

(Bernheimer *et al.*, 1980). This causes sufficient damage to cell membrane to cause cell lysis. The synergistic haemolysis is inhibitable by antiserum to either substance (Knight, 1978; Lund *et al.*, 1982a; Kuria, 1984). Inhibition of the haemolysis by antiserum to phospholipase D forms the principle of the synergistic haemolysis inhibition test, (Knight, 1978). All strains of *C. pseudotuberculosis* produce exotoxin albeit in varying amounts (Carne, 1940; Muckle and Gyles, 1982). *C. pseudotuberculosis* also produces a haemolysin, responsible for the narrow zone of ß-haemolysis observed in blood agar cultures. The haemolysin is therefore, a different substance from the phospholipase D (Carne, 1939). It cannot however be obtained free in culture supernant but is closely linked to the bacterial cells (Carne, 1939). The nature of the haemolysin has not been established. It has been suggested to be a phospholipase A (Soucek and Souckova, 1974).

C. pseudotuberculosis is said to produce an allergen, which can be harvested from cells grown in tripticase soy broth, by precipitation with trichloroacetic acid (Langenegger *et al.*, 1987). The allergen has so far not been characterised.

2.1.6. Pathogenicity of *C.pseudotuberculosis*.

Corynebacterium pseudotuberculosis causes suppurative lesions in a variety of animal species, including humans (Benham *et al.*, 1962; Blackwell *et al.*, 1974; Handerson, 1979; Keslin *et al.*, 1979; Goldberger *et al.*, 1981). The organism causes specific disease syndromes in sheep, goats, horses and to some extent, cattle. In sheep and goats, it causes caseous lymphadenitis, characterised by abscessation mostly of supperficial lymphnodes (Jubb *et al.*, 1985). In horses, the organism causes ulcerative lymphangitis. This is an excoriating suppurative inflammation of lymphatic vessels usually confined to the distal portions of the limbs (Gillespie and Timoney, 1981). In the same species, the organism has been found to

cause chronic ventral abscesses (Hughes and Biberstein, 1959; Miers and Ley, 1980), abortion (Miers and Ley, 1980) and mastitis (Addo *et al.*, 1974). In cattle the organism has been found to cause mastitis and abscesses located intradermally or within lymphnodes (Biberstein *et al.*, 1971; Adekeye *et al.*, 1980; Addo *et al.*, 1980; Kariuki and Poulton, 1982; Barakat *et al.*, 1984). Another domestic ruminant affected is the dromedary (Esterabadi *et al.*, 1975 Domenech, 1980;).

2.1.7. Mechanism of pathogenicity.

The pathogenic mechanism of *C. pseudotuberculosis* has been attributed to the surface waxy lipid and the extracellular exotoxin. The organism is a facultative intracellular parasite (Jolly, 1965c; Hard, 1972; Tashjian and Campbell, 1983). This ability is facilitated by the waxy lipid. The substance protects the organism from digestion by intracellular degradative enzymes (Hard, 1972). The substance also has toxic properties. It is dermonecrotoxic when injected intradermally (Carne *et al.*, 1956). Leucocytes in tissue culture fed with the substance adsorbed onto activated charcoal were found to undergo rapid degeneration and death (Carne *et al.*, 1956; Hard, 1975). Hard, (1972) and Tashjian and Campbell, (1983) examined the ultrastructure of mouse and goat macrophages respectively that had phagocytosed *C. pseudotuberculosis*. The organism was found to be viable within the phagolysosomes. Hard also observed damage to the phagolysosome limiting membrane. He attributed the damage to toxicity of a substance on the organism's surface. Similar ultrastructural damage to membrane components were observed in macrophages that had phagocytosed a solution of the waxy lipid extracted by petroleum/ether (Hard, 1975). Heat-killed *C. pseudotuberculosis* injected intraperitoneally in mice produced sterile abscesses (Zaki, 1976). From this observation, it was concluded that the waxy lipid was the pyogenic

factor of *C. pseudotuberculosis* and that it was heat-stable.

The amount of cell wall lipid in a *C.pseudotuberculosis* strain is related to it's virulence (Jolly, 1966; Hard, 1969a; Muckle and Gyles, 1983). Muckle and Gyles, (1983), inoculated 25 strains into mice intraperitoneally and noted that the strains with the greatest amount of lipid content, as determined by chloroform/methanol extraction, produced the most abscesses. Burrell, (1978a) similarly found a correlation between the lipid content of strains and their ability to produce lesions in popliteal lymphnodes of sheep. The cytotoxic effect of the waxy cell wall lipid appear not to affect rabbit phagocytes, which are able to rapidly degrade virulent organisms (Hard, 1975).

The extracellular toxin phospholipase D, is another important pathogenic factor of *C. pseudotuberculosis*. When injected subcutaneously in rabbits and guinea pigs, Carne (1940), found it to cause extensive, haemorrhagic necrotic lesions. Jolly (1965a), demonstrated that the exotoxin functions as a permeability factor. Injection of exotoxin intradermally caused oedema of interstitial spaces, swelling of endothelium and occasional thrombosis. When injected intraperitoneally in mice it caused increased protein exudation into the peritoneal cavity, and corneal opacity when injected into the avascular cornea in sheep. Jolly (1965a) concluded, from these observations, that the target site of phospholipase D was the vascular bed. Carne and Onon (1978), demonstrated that the exotoxin degraded sphingomyelins from sheep and rabbit aortae into ceramide phosphate and choline. They concluded that the exotoxin served the organism as a permeability factor.

2.1.8. Antigenicity of *C. pseudotuberculosis*.

C. pseudotuberculosis has a surface antigen that reacts with the homologous antibody to cause agglutination of bacterial cells. The antigen is also present in protoplasm but in smaller amounts. Cameron, (1972) found that the antigen is part of the cell wall matrix and that chemical alteration of the cell wall simultaneously destroyed the antigen. Shigidi, (1974) extracted a soluble antigen from bacterial cells using sodium desoxycholate that could be used for gel immunodiffusion tests. Two precipitin lines were observed when the antigen was reacted with antibacterial serum. Different strains of each biotype of *C. pseudotuberculosis* are antigenically identical with respect to their cell wall antigens (Shigidi, 1974; Awad *et al.*, 1977) but antigens from the two biotypes are different (Barakat *et al.*, 1984). The cell wall antigen cross-reacts slightly with that of *C. renale* but not with those of other *Corynebacteria* (Shigidi, 1974; Shen *et al.*, 1982).

The chemical component of the cell wall antigen is a non-protein and non-lipid as it is not affected by proteotytic enzymes (Cameron *et al.*, 1969; Cameron and Purdom 1971; Shen *et al.*, 1982), treatment with trichloroacetic acid (Cameron and Purdom, 1971) or extraction with ether/ethanol (Cameron and Fuls, 1973). The molecular structure and the molecular weight of the antigen has however not been determined.

The surface lipid of *C. pseudotuberculosis* is not antigenic (Maki *et al.*, 1985) and its removal by ether/ethanol does not alter the immunising ability of bacterial cells (Cameron *et al.*, 1972).

The exotoxin of *C. pseudotuberculosis* consists of several antigenic components. It reacts with the homologous antiserum to produce several precipin bands in agar gel (Goel and Singh, 1972; Kuria, 1984). These findings correlate with the chromatographic elution profile of the exotoxin which consists of several peaks that show activity (Goel and Singh,

1972; Onon, 1979; Kuria, 1984). The exotoxin is therefore thought to be an aggregate of more than one protein.

Exotoxin from different strains of both biotypes of *C.pseudotuberculosis* are antigenically identical (Doty *et al.*, 1964; Lovell and Zaki, 1966; Awad *et al*, 1977; Brown *et al.*, 1985) and do not cross-react with toxins of other *Corynebacteria* (Lovelll and Zaki, 1966; Soucek and Souckova 1974). *C. pseudotuberculosis* has also been found to produce an allergenic substance that provokes delayed skin hypersensitivity in infected animals (Langenegger *et al.*, 1987). The delayed hypersensitivity was specific in that it was not observed in healthy animals. There is no information as to whether the allergen is related to allergens of other intracellular bacterial parasites.

2.2. The disease in goats.

2.2.1. Epizootiology.

Caseous lymphadenitis is a disease mainly of adult animals. The disease is rare but can occur in kids. Ashfaq and Campbell, (1979) examined several herds and found only a few kids of 0-3 months infected. In adult animals, a direct correlation between age and prevalence was found. Animals of 3-4 years had the highest prevalence. Shen *et al.*, (1982) also observed a positive correlation between age and seropositivity in ELISA immunoassays. Holstad (1986c), however found no difference between different ages of adult animals (over 1 year) in the prevalence of superficial swellings in 15 herds. The prevalence was found to increase up to one year of age and was constant from then onwards. Lund *et al.*, (1982a) examined a naturally infected herd and found that animals were seropositive at the age of ten months. Holstad (1986a), followed the course of infection in 15 herds and found antibody titres to increase from one to six years of age. The increase was attributed to long-standing

antigenic stimulation.

Both males and females are equally affected (Ashfaq and Campbell, 1979) although a higher prevalence was observed in intact males than in castrates. It was suggested that the change in social behaviour was responsible for the difference in the sense that castrates are less aggressive and therefore less prone to injuries though which infection occurs. There is no information concerning the effect of breed on the prevalence of CLA in goats. In sheep, Nagy (1971) observed an apparently higher prevalence in merino sheep than in other breeds, with no apparent attributing factors.

2.2.2. The Sources of Infection.

Pus from discharging ruptured lymphnode or superficial abscesses contaminates fomites and the environment which then become the source of infection for other animals. *C. pseudotuberculosis* can survive in soil and on fomites for periods of up to one week and this ensures its continuing presence in the enviroment (Augustine and Renshaw 1982a; 1982b). The organism can also survive for periods ranging from three days to one week in water and on straw (Abdel-Hamid and Zaki, 1972). Nairn and Robertson (1974) demonstrated that contaminated dipping fluids can act as a source of infection for recently shorn sheep. Another potential source of infection is goat hair contaminated by direct contact (Ashfaq and Campbell, 1979; Brown and Olander, 1987). Holstad (1986b), observed that in 15 infected herds, the prevalence was lowest in a herd in which animals were individually kept in separate stalls. The purulent material has been estimated to contain 10^6 to 5×10^7 viable organisms per gram and so a single ruptured abscess can extensively contaminate the enviroment (Augustine *et al.*, 1982). Introduction of an abscessed animal is the most common source of infection for a disease free herd (Ashfaq and Campbell, 1979; Williamson and

Nairn, 1980; Holstad, 1986b). The disease, once established, becomes enzootic.

2.2.3. Route of Infection.

Most investigations into the routes of infection have focussed on sheep. In this species, contamination of superficial shearing wounds is believed to be the main route of infection (Nagy 1976; Nairn *et al.*, 1977; Brogden *et al.*, 1984). Transmission occurs when shears contaminated with pus deposits organisms on newly shorn, abraded skin (Nairn *et al.*, 1977). The disease can be reproduced experimentally by application of purulent material to fresh shearing wounds (Nagy, 1976) or by placement of culture on unbroken shorn skin especially when wet (Nairn and Rorbertson, 1974; Shigidi, 1979). Application of contaminated dipping fluids to skin within 2 weeks of shearing resulted in lesions in 3 out of 12 sheep (Nairn and Robertson, 1974). Nagy (1971), explored the possibility of arthropod vector (tick) transmission. However, here was no positive relationship found between tick infestation and prevalence of abscesses in sheep. Culturing mouth parts and saliva of ticks from 70 abscessed animals yielded *C. pseudotuberculosis* from only two ticks. Recovery of the organism from semen from rams with epididymitis have led to suggestions of possible venereal transmission (Mostafa *et al.*, 1973).

In goats, and in sheep that are not shorn, superficial skin wounds inflicted by thorns, barbed wire and other sharp objects or by fighting are thought to be the main portals of entry (Addo, and Eid 1978; Hein and Cargill 1981). Rubbing of the body against fence posts which could be contaminated with purulent material may be important in transmission (Burrell 1981). Ashfaq and Campbell (1979), considered that infection can occur through traumatised buccal mucosae due to rough feeds. This was borne out of observations that the most commonly affected lymphnodes in goats in western U.S.A. were those of the head region.

In sheep and goats with visceral form of CLA, a high prevalence of pulmonary and thoracic lesions has been observed. This has been taken to indicate possible respiratory transmission (Brown *et al.*, 1987). However, pulmonary abscesses can be produced experimentally not only through intratracheal but also through intravenous and intradermal inoculation (Cameron, 1972; Nagy, 1976; Brogden *et al.*, 1984; Brown *et al.*, 1985). It appears therefore that pulmonary infection is a result of either haematogenous or lymphogenous rather than aerogenous spread (Nairn and Robertson, 1974). Early pulmonary lesions were found to commence in the interstitial tissue without involvement of alveoli or bronchioles. The liver is thought to be affected via portal circulation and the infection can then affect the lungs (Benham *et al.*, 1962).

Experimentally, there are reports of caseous lymphadenitis induction by inoculation through scarified skin (Abdel-Hamid 1973), intradermal inoculation (Ashfaq and Campbell 1980), subcutaneous inoculation (Ashfaq and Campbell, 1980; Holstad and Teige, 1986b; Pepin *et al.*, 1988), intravenous, intralymphatic, intravaginal and oral routes (Cameron, 1972; Nagy, 1976; Burrell, 1978a; Brogden *et al.*, 1984; Holstad and Teige, 1988a). Intravenous inoculation usually leads to acute death, hence this route is unsuitable for experimental induction of CLA.

2.2.4. The dose of infection.

There is a wide variation in the reported dosage of *C. pseudotuberculosis* that will cause abscessation of regional lymphnodes with or without abscessation of internal organs. The dose varies depending on the route of inoculation and possibly the pathogenicity of *C. pseudotuberculosis* strains. Abdel-Hamid, (1973) inoculated 2 mls of a 48 hour broth culture into sheep subcutaneously and observed acute toxaemia. Doses of 0.25 mls and 0.01 mls

inoculated subcutaneously or by skin scarification with the same culture produced abscessation at inoculation site as well as the regional lymphnodes. Gameel and Tartour (1974), reported abscessation of regional as well as body lymphnodes in sheep inoculated subcutaneously with 1.9 x 10^5 organisms. A dose of 3.8 x 10^5 organisms produced acute death characterised by haemoglobinuria and icterus. Ashfaq and Campbell (1980), reported that a dose of 1 x 10^6 organisms delivered either subcutaneously, intradermally or submucosally in the mouth was suitable for experimental reproduction of CLA in goats. Holstad and Teige (1988b), used the same dose and achieved abscessation of regional lymphnodes in some animals and Maki *et al.*, (1985), reported a minimum dose of 1 x 10^4 organisms for infection in goats.

Pepin *et al.*, (1988), tried various doses inoculated subcutaneously in the outer ear of sheep and reported that at least 1 x 10^8 organisms were necessary to produce regional lymphnode abscesses. Some animals also became abscessed in the lungs. Depending on the dose of infection, intravenous inoculation leads to either no lesions, abscessation of visceral organs with or without involvement of visceral lymphnodes, or acute death (Cameron, 1972; Brown and Olander, 1987; Holstad and Teige, 1988a). Cameron, (1972), found difficulty in controlling the dosage that would give the desired lesions.

2.2.5. Pathogenesis.

From the primary site of infection, *C. pseudotuberculosis* disseminates as both free or phagocyte-borne bacteria and localise in lymphnodes and occasionally in a variety of internal organs (Jolly, 1965b; Hard, 1969b; Hard, 1972; Batey, 1974; 1986a; 1986b). Experimental evidence has shown that dissemination to visceral organs, especially lungs, occur concurrently with lymphnode infection (Nairn and Robertson, 1974).

Dissemination from the primary site is aided by the organism's phosphlipase D (exotoxin). The enzyme damages endothelial cell membranes, resulting in leakage of plasma from small blood vessels and capillaries and thereby increasing lymph flow (Carne and Onon, 1978). This facilitates spread of organism via lymphatics and haematogenous route. The activity of exotoxin has been demonstrated by the ability of antitoxin to inhibit exotoxin-induced vascular permeability in the skin of sheep, to reduce transfer of organisms to the local lymphnode and to decrease protein effusion following intraperitoneal challenge in mice (Jolly, 1965a). Zaki, (1976), found that the antitoxin localized infection to the primary site in mice infected intraperitoneally, while control mice developed abscesses throughout the body.

Bacteria disseminating through the haematogenous route are trapped and phagocytosed in lungs, liver or spleen while those disseminating via lymphatics are trapped in local and regional lymphnodes (Jolly, 1965b; , 1969). The eventual formation of lesions at primary or secondary sites is due to intracellular multiplication of bacteria which leads to rapid death of host cells (Jolly 1965b; 1965c; Hard, 1969b). The freed bacteria are subsequently engulfed by remaining or infiltrating phagocytes which again go through the process of degeneration and death. In an infected macrophage monolayer, Jolly, (1965c) found that macrophages underwent degeneration when the number of intracellular organisms reached 30 or 40. The freed bacteria were then engulfed by secondary cells and multiplication led to rapid formation of a plaque of dead and dying macrophages. In sheep, the cycle of phagocytosis, multiplication of organisms and cellular degeneration is the basis of the concentric rings arrangement of the pus in lymphnode lesions (Runnels *et al.*, 1967; Burrell, 1978a). The size of lesions formed varies but probably depend, on the initial number of organisms, the rate of multiplication and accessibility of lesion to host cells (Batey, 1986b). Although no direct

action of exotoxin on host phagocytes has been demonstrated, possible cytotoxicity to internal cellular membranes has been suggested (Burrell, 1978a).

Development of lesions does not always occur subsequent to infection. Burrell, (1978a) introduced *C. pseudotuberculosis* into afferent lymphatics of popliteal lymphnodes of sheep and found that typical lesions of CLA did not always follow but oedema was invariably present. In mice, Jolly, (1965b) found that resolution of hepatic microabscesses was common and that organisms could be recovered from spleen without presence of lesions. In affected sheep flocks, antibodies to exotoxin are not necessarily present or confined to sheep with lesions (Zaki and Abdel-Hamid, 1971; Nairn *et al.*, 1977; Shigidi, 1978; Burrell, 1980b). This suggests that resolution of lesions in some animals does occur in the early stages of development (Batey, 1986b).

2.2.6. The Clinical Disease.

Caseous lymphadenitis in both goats and sheep is characterised by one or more swellings involving the superficial lymphnodes. The swellings are either visually apparent or are detectable by palpation. The abscesses may burst, discharge and heal completely leaving a scar or no sign of infection (Addo and Eid, 1978). In Western United States, Ashfaq and Campbell (1979), reported that in goats, the most commonly affected lymphnodes are those of the head region as opposed to those of other parts of the body. The mandibular nodes had the highest incidence followed by the parotid. In Nigeria, Addo and Eid (1978), observed in goats and sheep a higher incidence in prescapular and precrural lymphnodes. The site of lesion is dependent on the site of infection. Nagy (1976), infected animals subcutaneously on different sites of the body and observed a definite relationship between infection site and the affected lymphnodes. Lymphnodes nearest to the site of infection were

affected first (Gameel and Tartour, 1974).

The disease occasionally becomes generalised with abscessation of internal lymphnodes and organs (Renshaw *et al.*, 1979, East, 1982). This form of the disease is less common but more severe. The clinical picture is indistinguishable from other chronic debilitating disease conditions in both goats and sheep. It has an insidious onset of wasting with occasional deaths (Gilmour, 1990). However, lesions of the retropharyngeal lymphnodes, bronchi or lungs will cause respiratory symptoms, choking and death (Addo and Eid, 1978; Holstad, 1986b). Chronically infected sheep have been found to die after a dibilitating disease characterised by anaemia (Gameel and Tartour, 1974). Renshaw *et al.*, (1979) observed a thinning syndrome in ewes due to chronic visceral CLA and Mostafa *et al.*, (1973), observed reproductive failure due to abscessation of lymphnodes draining the genitalia in ewes. Testicular infection has been incriminated in infertility in a ram (Turner, 1980).

Blood parameters in infected animals have been examined. In both naturally and experimentally infected animals, there is usualy a leucocytosis and also enaemia (Addo and Eid, 1978; Mottelib *et al.*, 1979; Holstad and Teige, 1988b). The anaemia is characterised by a drop in haemoglobin and haematocrit levels, and this is thought to be due to toxic effects of exotoxin on erythrocytes (Cameron *et al.*, 1972).

2.2.7. Pathology.

At post mortem, apart from superficial and internal lymphnodes, other organs that may be found affected are the liver, lungs, kidney, spleen and subcutaneous tissue (Sharma and Dwivedi, 1976; East, 1982; Jubb *et al.*, 1985). In sheep, lesions have also been encountered in the brain (Bandopadhyay *et al.*, 1976), in the heart (Hamir, 1981), scrotum

(Williamson and Nairn, 1980), and epididymis and testicular tissue (Krishna *et al.*, 1977). Lesions in the lungs may be either descrete abscesses or a diffuse suppurative bronchopneumonia (Sharma and Dwivedi, 1976; Addo and Eid ,1978).

The abscess material is usually greenish-yellow to creamy in colour and pasty in consistency. The pasty nature of pus in goats is said to be distinct from the onion-like concentric rings of pus arrangement in sheep (Brown and Olander, 1987). This is thought to be due to a more liquefactive nature of goat phagocytic enzymes (Ashfaq and Campbell, 1980). Calcifacation of the lesions is reportedly rare in goats (Brown and Olander, 1987).

Microscopically, lesions are granulomatous and resemble tubercles. A caseous necrotic centre, with fragmented nuclear debris and degenerated and intact neutrophils is surrounded by a zone of macrophages, lymphocytes, epitheliod, plasma and giant cells. The lesion is encircled by a capsule of proliferating fibroblasts (Sharma and Dwivedi, 1976; Jubb *et al.*, 1985). Gram-positive diphtheroid organisms may be observed in the necrotic zone of the lesion.

2.3. Immune Response.

2.3.1. Tests for Humoral Response.

These have been adequately reviewed by Ayers, (1977); Kuria, (1984); and Brown and Olander, (1987). The tests can be grouped into those detecting antibody to cell surface and cell wall antigens and those detecting antibody to exotoxin. The earliest of the first category was the bacterial agglutination test (BAT). The test detects antibodies to surface antigen. Originally performed in tubes, (Cameron and McOmie, 1940; Awad, 1960) BAT is today performed in microtitre plates (Lund *et al.*, 1982a; Holstad, 1986a). The tendency of *C. pseudotuberculosis* to autoagglutinate presents a problem in antigen preparation.

However, some strains have less tendency to autoagglutinate (Jolly, 1966). Autoagglutination can also be inhibited by detergents such as Tween 80 (Keskintepe, 1976b; Lund *et al.*, 1982a). The test has been reported to have a sensitivity and specificity of 96% in experimentally infected animals (Holstad, 1986a) but non-specific agglutination reactions have been reported (Burrell, 1978b).

An agar gel double immunodiffusion test is performed using a soluble antigen extractable from bacterial cells using sodium desoxycholate (Shigidi, 1974). The test shows some non-specific reactions which were attributed to possible cross-reactions (Shigidi, 1979). The antigen is known to cross react with an antigen of *Corynebacterium renale* (Shigidi, 1974). A cell wall antigen ELISA has been established. It utilises a trypsinised particulate cell wall antigen separated from sonicated cells using a density gradient (Shen *et al.*, 1982). The test does not cross-react with antigens of *Corynebacterium (Actinomyces) pyogenes.*

Tests for detection of antitoxin are either *in vivo* or *in vitro* tests. A skin neutralisation test, in which exotoxin is titrated with antiserum and then injected intradermally in rabbits has been attempted (Doty *et al.*, 1964). Absence of oedema and necrosis indicates presence of antibodies. The test is not applicable in large scale testing. Zaki (1968), utilised the ability of exotoxin to inhibit erythrocyte lysis by *Staphylococcal* ß-lysin to develop the anti-haemolysin inhibition test (AHI). Test serum is incubated with standard dose of exotoxin and bovine erythrocytes after which *Staphylococcal* ß-lysin is added. In the absence of antibodies, exotoxin occupies receptor sites on erythrocyte membrane and prevents ß-lysin from occupying them and causing haemolysis. The test was reported to have a sensitivity and specificity of 92-96% in an abattoir survey of the disease in sheep and goats (Zaki, 1968).

The haemolysis inhibition test (HIT) or synergistic haemolysis inhibition test (SHI) is based on the synergistic action of exotoxins from *C. pseudotuberculosis* and *Rhodococcus*

equi to cause erythrocyte lysis (Fraser, 1964). Antibodies to exotoxin inhibits the synergistic haemolysis. The test was first applied in infected horses and was reported to have a sensitivity of 100% (Knight, 1978). Titres are said to remain positive even in the subclinical stage of infection in goats, indicating that the test can detect infected carriers (Brown *et al.*, 1986b; 1987). The sensitivity of the test has been reported as 96% in experimental goats and as 98% and 96% respectively in sheep and goats with natural disease (Holstad, 1986a; Brown *et al.*, 1986b).

An antitoxin ELISA has also been developed (Maki *et al.*, 1985; Kuria and Holstad, 1989) and found superior to ELISA systems detecting antibodies to other antigens of *C. pseudotuberculosis.* An agar gel immunodiffusion test or an indirect haemagglutination test can also be used to detect serum antitoxin (Burrell, 1980a; 1980b).

Another *in vivo* test is the mouse protection test (Zaki and Abdel-Hamid, 1971). Test serum is titrated with standard amounts of exotoxin and then injected intraperitoneally in mice. Presence of antitoxin in serum is indicated by survival of mice.

2.3.2. Humoral Immune Response in CLA.

Bacterial agglutination antibodies are detectable by the second week of infection in experimental animals (Holstad and Teige, 1988b). Antibody titres increase during the first two weeks and then decrease subsequently for a period of two to three weeks. Cameron (1972), found bacterial agglutination titres to return to pre-immunization levels within four months. In the cell wall antigen ELISA, Shen *et al.*,(1982) observed a significant rise in titres in the first two weeks post inoculation. The rise peaked at 5 weeks and then declined slightly. Antibodies to exotoxin are detectable about one month post infection (Abdel-Hamid and Zaki, 1973; Brown *et al.*, 1985; Maki *et al.*, 1985; Holstad and Teige, 1988b).

Antibodies to cell wall antigens are therefore detectable earlier than antitoxin. Shigidi (1979), compared five tests, (three detecting antibodies to cell wall antigens and two detecting antitoxin), in experimentally infected sheep. A wide variation was observed between the tests in their onset and duration of sensitivity. The animals had been infected by placing the organism on intact shorn skin. This method of infection is largely ineffective in establishing caseous lesions. Antibodies to *C. pseudotuberculosis* are transferred to new borns through colostrum. Lund *et al.*, (1982b) found serum from kids suckled by infected nannies to have antitoxin titres detectable up to 5-6 weeks of age and not later than $2^1/_2$ months. The significance of these observations was that seropositivity in kids after this period indicate exposure to *C. pseudotuberculosis* antigens. Kids are also protected, to an extent, from infection by maternal antibodies up to that period.

2.3.3. Tests for Cell-mediated Immune Response.

Work done on cell mediated immune response has focussed mainly on delayed skin hypersensitivity tests. *C. pseudotuberculosis* is a facultative intracellular parasite. A host's cell mediated immune response is therefore expected. Tuberculin has been found to elicit non-specific reactions in sheep and goats naturally and experimentally infected with *C. pseudotuberculosis*. The reactions are probably due to antigens shared with *Mycobacterium spp*. (Shukla *et al.*, 1971). Train (1935), and Cameron and McOmie (1940), attempted delayed skin hypersensitivity tests in horses and sheep respectively using *C. pseudotuberculosis* culture filtrate. The results were highly variable. Renshaw *et al.*, (1979) used sonicated organism as the test reagent. The sensitivity of the test was found to be low (56%). Brown *et al.*, (1986a) injected fragmented cells at regular intervals into experimentally infected goats. A hyposensitivity response was observed and was found to

have a direct relationship with the severity of the clinical disease. Langenegger *et al.*, (1987) produced an allergen from *C. pseudotuberculosis* cells. The allergen reportedly produced specific skin reactions in 40 infected goats and no reactions in 40 normal goats. There are few reports of invitro tests for cell medicated immune response. A lymphocyte transfer test has been attempted but the results were found highly variable and unreliable (Holstad *et al.*, 1989).

2.3.4. Cell Mediated Immune Response.

C. pseudotuberculosis is a facultative intracellular parasite and cell mediated immune response is expected to play an important part in immunity to CLA. Jolly, (1965b) observed that resolution of lesions in livers and spleens of convalescent mice was by specialised macrophages. Also, an emergence of activated macrophages has been observed following infection and on re-infection (Jolly, 1965b; 1965c; Hard, 1969b; 1970). Immunity can also be transferred through peritoneal cells from mice infected intraperitoneally to non-infected mice (Hard, 1969b). These findings indicate that cellular immunity play a major role in resistance to *C. Pseudotuberculosis* infection. Cellular immune response has also been demonstrated by Husband and Watson, (1977). Live *C. pseudotuberculosis* injected intralymphatically into afferent lymphatic ducts increased the number of lymphoblasts in efferent lymph four times higher and for four days longer than the killed organism.

Investigations have been done concerning possible depression of immune response by *C. pseudotuberculosis* through destruction of lymphoid tissue. Hedden *et al.*, (1986) characterised lectin-binding lymphocytes of goats with and without CLA. Results indicated that goats with CLA may have compromised cell mediated immunity.

3.0 MATERIALS AND METHODS.

3.1. Natural pseudotuberculosis in goats and sheep.

3.1.1. Animals.

Animals slaughtered in the Nairobi municipal slaughterhouses of Kitengela, Dandora, Halal and Dagoretti were used. These animals came from different parts of the country but mainly from the nomadic North Eastern, Eastern and Rift Valley provinces. The breeds of goats were mainly the Galla and the small East African goat while sheep breeds were the dopper, blackhead Persian, Red Maasai and crossbreeds. The animals were examined for pseudotuberculosis lesions at postmortem.

3.1.2. Postmortem Examination.

The lymphnodes of the head and body and those of the visceral organs were incised and examined for abscesses. The visceral organs were examined by palpation and incission. Any abscessed lymphnode or tissue was put into separate sterile plastic containers and transported to the laboratory.

3.1.3. Bacteriological analysis.

Caseous pus from abscessed lympnode or other tissues was cultured on 8% ox blood agar and incubated at 37°C for a maximum of 48 hours. Bacterial colonies were then identified following the standard procedure i.e physical characteristics, cellular morphology, staining characteristics of cells and biochemical reactions (Collins and Cummings, 1986).

3.1.4. Histopathology.

Samples of abscessed tissues were fixed in 10% neutral buffered formalin, processed

routinely by paraffin block method, and then sectioned at 5 μm thickness. All sections were stained with haemotoxylin and eosin (H&E). Sections that showed signs of calcification were also stained with Von Kossa stain to demonstrate the presence of calcium compounds (Luna, 1968).

3.1.5. Serology.

Blood or blood clots were obtained from heart chambers of affected and non-affected carcasses, put into sterile universal bottles transported to the laboratory, and processed for serum. The serum samples were preserved with sodium azide (0.01%) and stored at -20°C until used for serological tests, namely the haemolysis inhibition test (HIT) and the bacterial agglutination test (BAT).

3.1.5.1. The haemolysis inhibition test (HIT).

The reagents required for this test were *C. pseudotuberculosis* exotoxin, cell-free culture filtrate of *Rhodococcus equi*, sheep red blood cells (srbc) and diluent.

(a) Preparation of *Rhodococcus equi filtrate*.

Rhodococcus equi (*Rh. equi*) filtrate was prepared from strain NVH 3370,(Norwegian College of Vet. Medicine Culture Collection). The organism was cultured in 3.7% Brain-heart infusion broth (Oxoid) and incubated at 37°C with continuous shaking for 5 days (Lund, 1982a). The culture was centrifuged at 14 x 10^4 x g for 30 minutes at 4°C. The supernatant was filtered through 0.22 μm size millipore filters (Millipore corporation), preserved with merthiolate (final concentration 0.01%00), and stored at 4°C.

(b) Preparation of *C. pseudotuberculosis* exotoxin.

C. Pseudotuberculosis exotoxin was prepared from a local strain HL13, previously isolated from a caseous abscess in a goat. The organism was cultured in 500 mls volumes of 3.7% Brain-heart infusion (oxoid) broth incubated statically at 30°C for 7 days. Cultures were then centrifuged at 14 x 10^4 x g for 30 min. at 4°C. The supernatant was filtered through 0.22 μm size millipore filters, preserved with sodium azide (final concentration 0.01%) and stored at 4°C.

(c) Preparation of sheep red blood cells.

Blood was collected from the jugular vein of a sheep, mixed with an equal volume of Alserver's citrate and left to stand overnight at 4°C. The buffy coat was removed and the red cells washed three times with Phosphate Bufferd saline (0.15M PBS, pH 7.2) The cells were stored in Alserver's citrate at 4°C and used within one week.

(d) Titration of *C. pseudotuberculosis* exotoxin and *Rhodococcus equi filtrate*.

Exotoxin activity was measured in minimum haemolytic doses (MHD). One MHD of exotoxin was the concentration in 50 ul required to haemolyse 50 μl of 1% sheep red blood cells (srbc) by a mixture of the exotoxin and cell-free culture filtrate of *Rh. equi* (Knight, 1978). This was determined by a checkerboard titration. Briefly, serial two-fold dilutions of exotoxin in PBS were made in wells of a V-shaped polystyrene microtitre plate (Limbro), from left to right in 50 μl amounts. Serial two- fold dilutions of *Rh. equi*, in 50 μl amounts, prepared in a separate plate, were added into the wells , from top to bottom. Fifty microlitres (50 μl) of srbc (1% V/V) in PBS were then added into each well. The plate was then sealed, shaken, and incubated at 37°C for 24 hours. The highest dilution of

exotoxin that produced complete haemolysis of the srbc in the presence of the highest dilution of *Rh. equi* filtrate was taken to contain one MHD. The corresponding dilution of *Rh. equi* filtrate was taken to be the minimum concentration for the synergistic haemolysis.

(d) The serological test procedure.

The test was carried out as described by Lund *et al.*,(1982a). Two-fold serial dilutions of test serum in PBS were prepared in wells of V-shaped microtitre plates. Fifty microlitres (50 μl) of 2 MHD concentration of exotoxin was added into each well. The plates were incubated at 37°C for 2 hours. Fifty microlitres of a mixture of the optimum *Rh. equi* filtrate and sheep Rbc (1%) in PBS was added into each well. The plates were sealed, shaken, and incubated at 37°C for 24 hrs. Titres were read as the reciprocal $\log_{10}$ of the highest dilution of serum that inhibited haemolysis. Titres equal and above 0.6 were considered positive (Lund *et al.*, 1982a).

3.1.5.2. Bacterial agglutination test (BAT).

The reagents required for this test are:- test serum; whole bacterial cell suspension antigen and diluent.

(a) Preparation of antigen.

The antigen was prepared following the method of Lund, (1982a). A non-autoagglutinating local strain of *C.pseudotuberculosis* (HL10) was used. The strain had been isolated from a caseous abscess in a lymphnode from a goat and preserved by lyophilisation. The organism was cultured in 100 ml volumes of 3.7% Brain-Heart infusion broth containing 1% Tween 80 and incubated at 37°C with continuous shaking. Bacterial cells were washed

three times with PBS containing 1% Tween 80 (PBS- Tween) and then suspended in the same buffer to a concentration of optical density (O.D) 0.65 ± 0.05 at 540 nm wavelength. This constituted the agglutinating antigen.

(b) The serological test.

Two-fold serial dilutions of test serum in PBS were prepared in wells of U-bottomed polystyrene microtitre plates (Limbro). Fifty microlitres of the agglutinating antigen was added into each well. Plates were sealed, shaken and incubated at 37°C overnight. Titres were read as the reciprocal $\log_{10}$ of the highest serum dilution that agglutinated the antigen. Titres equal and above 2.1 were taken as positive (Holstad, 1986a).

3.1.6. Statistical analysis.

The sensitivity and specificity of serological tests were calculated by means of a Chi-square (Steel and Torrie, 1980) as follows:-

		Presence of Lesions.	
		+	-
Serological	+ve	a	b
Reaction.	-ve	c	d

$$\text{Sensitivity} = \frac{a \times 100}{a+c} \qquad \text{Specificity} = \frac{d \times 100}{b+d}$$

The distribution of titre values among infected and none- infected animals was analysed by analysis of variance (Steel and Torrie, 1980).

3.2. Experimental induction of lymphadenitis.

This was carried out through investigation on different routes of infection, the dose required to set up an infection, the incubation period for the formation of lesions, and the ability of *C. pseudotuberculosis* surface lipid, the exotoxin and whole cell preparations to produce caseous lesions.

3.2.1. Routes of infection.

3.2.1.1. Experimental animals.

Experimental goats were of the Small East African breed, obtained from Kisamis area of Kajiado District. Castrated males of approximately 6 months of age and approximately of equal weight were selected and held in one *boma*. They were screened for pseudotuberculosis by physical examination (palpation) of superficial lymphnodes and by serological examination using the BAT and HIT. Fourteen of those found negative were purchased and used for the experiment.

The animals were given prophylactic treatment with 2 mls long acting tetracycline each intramuscularly, dewormed with 1.5 mls of albendazole, 10% (Valbazene(R), Kenya Swiss Co. Ltd.) and also sprayed for ectoparasites with acaricide (Amitraz(R) 12.5% W/V, Cooper (Kenya) Ltd. Acaricide and deworming treatments were repeated after 1 week and 2 weeks respectively.

The animals were housed in concrete-floored, stone-walled stalls with straw bedding. They were fed on hay and a wheat bran/dairy meal mixture. Animals were allowed 2 weeks

to acclimatise. They were again screened for pseudotuberculosis before commencement of experiment.

3.2.1.2. Preparation of infectious caseous abscess material.

Caseous abscess material was produced by injecting subcutaneously into a rabbit 0.1 ml of 19 hour brain-heart infusion culture of *C. pseudotuberculosis* strain HL13, emulsified in incomplete freund's adjuvant. The purpose of using incomplete Freund's adjuvant was to contain the organism at the injection site. Two weeks later, the fur around the ripe abscess was shaved, the area cleaned with soap and water and disinfected with 70% alcohol. The abscess was then lanced and the caseous abscess material squeezed into sterile universal bottles.

3.2.1.3. Determination of bacterial concentration in abscess material.

The bacterial concentration in the abscess material was determined by the plate count method using the surface drop technic (Miles, 1938, Baker and Breach, 1980). A 10% (W/V) suspension of the material in Ringer's solution was prepared, thoroughly mixed on a whirlpool mixer and centrifuged at a low speed to remove fibrous material. Two- fold serial dilutions of the supernatant were prepared in the same diluent and 100 μl volumes of dilutions 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} plated on ox blood agar. After 48 hours incubation at 37°C, colonies were enumerated. The viable bacterial concentration, expressed in colony forming units (cfu), of the abscess material was calculated from the mean count from four platings of each dilution.

3.2.1.4. Infection procedure.

On each of the 14 experimental goats, an area approximately 2.5 sq cm and approximately 5 cm behind the right precrural (subiliac) and the prescarpular lymphnodes was shaven clean and disinfected with 70% alcohol. The animals were then divided into 5 groups of 3, 3, 3, 3, (Groups 1-4 and 2, (group 5) respectively and infected on both sites as follows:-

Group 1. Animals were injected subcutaneously with 100 μl of diluted pus containing 1×10^6 cfu of *C.pseudotuberculosis.*

Group 2. Animals were injected intradermally with 100 μl of diluted pus containing 1×10^6 cfu of *C. pseudotuberculosis.*

Group 3. The prepared sites were superficially scarified with sterile scapel blades and approximately 40 mg of undiluted pus smeared onto the sites using sterile spatulas.

Group 4. Undiluted pus, 40 mg, was smeared on the intact skin.

Group 5. This served as the unifected control group.

In groups 1-4, the right prescapular and precrural lymphnodes were the test nodes while the contralateral lymphnodes served as controls in each animal. The infection procedure is summarised in table 1.

Table 1.

Infection procedure of goats with *C.pseudotuberculosis* caseous abscess material through different routes.

Group.	Goat No.	Route of Infection.	Dose.
I.	1, 2, 12	subcutaneous	1×10^6 cfu.
II.	5, 6, 7	intradermal	1×10^6 cfu.
III.	8, 10, 11	scarified skin	caseous pus
IV.	9, 13, 14	intact skin	caseous pus
V.	3, 4	None	None

3.2.1.5. Clinical, haematological, serological and postmortem examination of the animals

Rectal temperatures were taken daily for one week. The animals were examined daily for swelling at infection sites, and draining lymphnodes and discharges from infection sites. Blood was collected weekly for haematology and for serum extraction. For haematology, the total white blood cell (WBC) count ($10^3/mm^3$) was determined using a coulter counter

model ZM equipment (coulter electronics, England); haemoglobin (g/100 mls) with a haemoglobinometre (coulntronics, France); the total protein concentration (g/100 mls) with a refractometer (Atago, Japan), and the packed cell volume (PCV) was determined using the capillary tube method and the Hawksley microhaematocrit reader. The differential leucocyte counts were calculated from Giemsa - stained smears. Serology was performed, using the BAT and HIT tests on all the sera collected as in section 3.1.5. All animals were sacrificed (slaughterd) at 10 weeks post infection and postmortem performed. The superficial and internal lymphnodes and visceral organs were examined for caseous abscesses. Bacteriological analysis was performed on all abscesses found. The right and left prescapular and precrural lympnodes were excised, striped of all extracapsular tissues, weighed and then fixed in 10% buffered formalin. They were then processed routinely for histological examination as described in 3.1.4.

3.2.6. Statistical analysis.

Changes in haematological values within and between groups were analysed by analysis of variance and by a Scheffe's test for separation of means. Lympnode weight differences were analysed by a T-test and serological results by analysis of variance (Steel and Torrie,1980).

3.3. Determination of the minimum dose of Infection.

3.3.1. Infection procedure.

The minimum dose of *C. pseudotuberculosis* required to establish caseous lesions in draining lympnodes was determined by infecting animals intradermally with graded doses of the organism.

Sixteen (16) goats weighing 12-18 kg were used. They had been tested and selected as in 3.2.1. They were divided into 8 groups of 2 animals each. Medication housing and feeding was carried out as described in 3.2.1.1.

The right prescapular and precrural sites were prepared as described in section 3.2.1.4. The draining lympnodes were the test lymphnodes and the contralateral lymphnodes the controls in the experiment. Infections was then carried out intradermally with 100 μl of caseous abscess material diluted in Ringer's solution to the required bacterial concentration as tabulated in Table 2.

Table 2.

Determination of the dose of infection for CLA in goats using caseous abscess material containing different doses of *C. pseudotuberculosis* administered intradermally.

Group	Goat No.	Dose of Infection (cfu)
1	15, 16	1×10^5
2	17, 22	5.0×10^4
3	18, 19	1×10^4
4	27, 21	5.0×10^3
5	29, 30	1×10^3
6	23, 24	5.0×10^2
7	31, 32	1×10^2
8	25, 26	5.0×10^1

3.3.2. Examination of animals.

Animals were observed and examined clinically daily for swelling of test and control lymphnodes and swelling and discharges at injection sites. Rectal temperatures were also taken daily. Blood for haematology and serology was collected weekly and analysed as in section 3.2.1.5.

3.3.3. Post mortem Examination.

All animals were sacrificed 10 weeks post infection and postmortem performed. The superficial and internal lymphnodes and visceral organs were examined for caseous abscesses. Test and control lymphnodes were weighed and then fixed in 10% buffered formalin. Thereafter they were re-examined for abscesses by thin- sectioning throughout the length of the nodes. Histopathology was latter performed as described in section 3.1.4.

3.3.4. Statistical analysis.

Haematological results were analysed by analysis of variance and a Scheffe's test for separation of means. Serological results were analysed by analysis of variance and lympnode weights by a T-test (Steel and Torrie, 1980).

3.4. Determination of the incubation period for CLA.

The incubation period for the formation of caseous lymphadenitis lesions in draining lymphnodes was determined by intradermal infection followed by sequential sacrifice and examination of three groups of goats.

3.4.1. Infection procedure.

Three groups of 10 goats each, tested, selected, medicated, housed and fed as in section 3.2.1.1. were used for the experiment. The right prescapular and precrural sites were prepared as described in section 3.2.1.4. The contralateral lymhnodes were the controls in each animal. The infection procedure was carried out as follows:-

Group 1. Animals were infected intradermally with 100 μl of diluted caseous pus containing 5.0×10^4 cfu of *C. pseudotuberculosis.*

Group 2. Animals were infected intradermally with caseous material containing 1×10^5 cfu of *C. pseudotuberculosis.*

Group 3. Undiluted caseous pus, 40 mg, was smeared on scarified sites using sterile spatulas.

3.4.2. Examination of animals.

The animals were examined physically for swelling of test lymphnodes and infection sites. Concurrently, animals from each group were sacrificed initially two-three days in groups one and two, and every two days in group three as follows:-

	Days animals were sacrificed.
Group 1.	2, 5, 7, 9, 11, 14, 17, 20, 27, 30.
Group 2.	2, 3, 3[*], 6, 8, 11, 17, 25, 34.
Group 3.	5, 7, 9, 11, 13, 15, 17, 19, 21, 23.

[*]The animal died of peumonia.

Blood for serology was collected from each animal at day zero and at time of sacrifice. In addition, animals remaining in group two after each sacrifice were bled every three days. BAT and HIT tests were performed as in section 3.1.5.

At postmortem, the test and control lymphnodes were thin-sectioned and examined for abscesses. Internal lymphnodes and organs were similarly examined. Sections of test and control lymphnodes and skin injection sites were fixed in 10% buffered formalin and processed for histological examination as in section 3.1.4.

3.5. The pathogenicity of *C. pseudotuberculosis* fractions and whole cell preparations.

The ability of two fractions (the surface lipid wax and the exotoxin) and three whole-cell preparations (formalin or heat killed or ether/ethanol washed) of *C. pseudotuberculosis,* to produce caseous skin and lymphnode lesions in goats was investigated. This was carried out by intradermal injection of each test substance followed by clinical, postmortem and histopathological examination of the injection sites and draining lympnodes.

3.5.1. Preparation of surface lipid wax.

C.pseudotuberculosis strain HL13 was grown on ox blood agar plates and incubated for 48 hours at 37°C. Colonies were then washed off with sterile PBS. The cells were washed three times with sterile distilled water, placed into 50 ml beakers and dried out for two days in an oven at 45°C. The cells were then weighed and wax extracted with diethyl ether following using the soxhlet method. The heating waterbath was maintained at 45°C.

3.5.2. Praparation of formalin - killed *C.pseudotuberculosis.*

Cells of *C. pseudotuberculosis* strain HL 13 grown on ox blood agar were washed three times with PBS and then suspended in PBS containing 12.5% of 40% formaldehyde solution (to make the final concentration of formaldehyde 5%). The cells were left to stand in the solution for two days. They were then washed with PBS containing 0.5% formaldehyde (final concentration) and then suspended in the same to a concentration of 5% cells (v/v)

3.5.3. Preparation of Heat-Killed *C.pseudotuberculosis.*

Washed *C.pseudotuberculosis* strain HL13 cells were suspended in PBS to a concentration of 5% cells (v/v) and then autoclaved at 121°C for 15 minutes.

3.5.4. Preparation of ether/ethanol extracted cells.

C. pseudotuberculosis cells were extracted three times for 2hrs. each with a 50:50 diethyl ether/ ethanol mixture. Cells were placed in 100 ml glass-stoppered flasks with glass beads added. Extraction was done on a mechanical shaker at room temperature. The cells

were then dried at 40°C and suspended in PBS to a concentration of 5% cells (v/v).

3.5.5. Purification of exotoxin.

C. pseudotuberculosis exotoxin was prepared as in section 3.1.5.1.(b). Three litres of the toxic brain-heart infusion broth with approximately 66 mg protein per ml (determined by the method of Lowry *et al.*,1982), was concentrated to 400 mls by ultrafiltration using a 30,000 daltons molecular weight retention filter(PM30, Diaflo ultrafilters, Amicon, Massachusetts, USA) at a filtration pressure of 70 psi.

The concentrate (300 mls) was precipitated with 700 mls of saturated ammonium sulphate solution (pH 8.0) to give a salt concentration of 70% in the mixture. The mixture was held at 4°C for two days and the supernatant decanted. The remainder of the solution was centrifuged at 14 x 10^4 xg. The precipitate was dissolved in 10mls of 0.2M Tris-HCl buffer, pH 8.0. Further purification was done by gel filtration.

Three (3) mls of the dissolved precipitate, containing 150 mg of protein per ml, were applied to a 1.5 cm diameter and one metre long column, packed with fine sephadex G50 (Pharmacia fine chemicals, Uppsala, Sweden) in Tris-Hcl buffer and eluted with the same buffer. The column was eluted at the rate of seven (7) mls per 10 min (about 40 mls per hr) and fractions of seven mls each were collected. The optical density (O.D) of each sample was read at 280 nm and at 410 nm. All samples were then tested for synergistic haemolytic activity on sheep red blood cells mixed with *Rh. equi* filtrate. Fractions that had the peak haemolytic activity were concentrated by ammonium sulphate precipitation, dialysed against a solution of 50% glycerol in Tris-Hcl buffer and stored at -20°C.

3.5.6. Pathogenicity testing procedure.

Groups of two to six goats were prepared for testing by shaving and disinfection of both the right and left precrural and prescapular regions as described in section 3.2.1.4. Injections were then carried out intradermally as follows:-

1. Animals, (six) were injected on the right side with 100 μl of an emulsion containing *C. pseudotuberculosis* lipid wax (20 mg), activated charcoal (5 mg), emulsified in liquid paraffin (0.5 mls). The activated charcoal acted both as an adsorbant and a tracer while the liquid paraffin served as a diluent as well as carrier. The contralateral side was injected with an emulsion of activated charcoal in liquid paraffin.

2. Animals, (four) were injected with 100 μl on the right side with a mixture of *C.pseudotuberculosis* exotoxin (100 μl) of approximately 50 mg of protein per ml and 4096 MHD strength per 50 μl and activated charcoal (5 mg) emulsified in incomplete Freund's adjuvant (0.5mls). The purpose of the activated charcoal was to adsorb the toxin and also act as tracer while that of the incomplete Freund's adjuvant was to bind and prevent diffusion the liquid toxin. On the left, 100 μl of an emulsion of saline and activated charcoal in the adjuvant was injected.

3. Preparations as (2) above but with heat-inactivated toxin on the right side in four animals.

4. Formalin- killed *C.pseudotuberculosis* preparation (100 μl) (see section 3.5.2.), on the right side only in two animals.

5. Heat- Killed *C.pseudotuberculosis* preparation (100 μl) (see section 3.5.3.), on the right side only in three animals.

6. Ether/ ethanol extracted *C.pseudotuberculosis* cells preparation (see section 3.5.4.) (100 μl), on the right side only in three animals.

3.5.7. Clinical, serological and postmortem examination.

The injection sites and the draining lympnodes were examined daily. Animals from each group were sacrificed on different days (days 6, 9, 24, 30, 35 and 40 for group one; days 9, 24, 30 and 35 for groups two and three; days 24 and 30 for group four; days 12, 24 and 36 four group five; and days 18, 21 and 30 for group six) postmortem carried out. Blood for serology (BAT and HIT tests) was collected from each animal at day zero and at time of sacrifice. The test (right) and the contralateral lympnodes were examined for lesions. The skin injection sites were also incised and examined. The lymphnodes were weighed and together with the skin injection sites, fixed in 10% neutral buffered formalin and processed for histopathological examination as described in section 3.1.4.

4.0. RESULTS.

4.1. Natural pseudotuberculosis in goats and sheep.

4.1.1. Gross pathology.

Seven hundred and fifty seven (757) goats were examined from the various slaughter houses. Of these, 54 carcasses (7.1%) had abscesses in one or more locations. A total of 56 abscesses were encountered. Of these, 50 were exclusively in peripheral lymphnodes (40 in prescapular, 6 in precrural, 3 in parotid and 1 in submaxillary), 2 involved precrural lymphnodes as well as the surrounding subcutaneous tissues, and 4 were in subcutaneous tissue, close to but with no involvement of peripheral lymphnodes (3 close to prescapular and one close to a precrural lymphnode) (Table 3).

Of the 398 sheep examined 6 carcasses (1.8%) had abscesses in one or more locations. In all, 7 abscesses were observed. Three of these were in prescapular lymphnodes, one was in a precrural lymphnode, 2 in subcutaneous tissue close to but with no involvement of a prescapular and a precrural lymphnode and one was in the lung parenchyma (Table 3). Other organs in both species were normal in appearance.

Lymphnode abscesses were greenish yellow or creamy in colour. The consistency was either caseous, granular or hard and calcified. One caseous lymphnode from a sheep had the pus arranged in onion - like concentric circles. Subcutaneous abscesses were greenish yellow and caseous. None was found to be calcified. Lymphnode abscesses occupied either

Table 3.

The distribution of caseous abscesses in sheep and goats with natural pseudotuberculosis observed during postmortem inspection in various slaughterhouses around Nairobi.

	No. of Abscesses.			
Location of Abscess.	**Sheep.**	**%**	**Goats.**	**%**
Prescapular Ln*.	3	42.85	40	75
Precrural Ln.	1	14.28	8	10.7
Parotid Ln.	0	0.0	3	5.35
Submaxillary Ln.	0	0.0	1	1.78
Lung.	1	14.28	0	0.0
Subcutaneous tissue.	2	28.57	4	7.14
Total	7	99.98	56	99.97

*Ln = Lymphnode.

the entire node, one pole, or occurred as multiple foci distributed within the lymphnode parenchyma.

4.1.2. Histopathology.

Lymphnode lesions of both goat and sheep consisted of a necrotic centre of pink-staining material containing nuclear debris, surrounded successively by a zone of macrophages and polymorphonuclear granulocytes (PMN), a zone of epithelioid and giant cells, macrophage together with lymphocytes and plasma cells and lastly a peripheral zone of fibroblasts or fibrous tissue (Fig.1). The number of giant cells varied from numerous to scanty. The capsular layer also varied from a loose layer of proliferating fibroblasts to a dense layer of fibrous tissue (Fig.2). Some lymphnodes had more than one lesion (Fig.3). In the rest of the lymphnode tissue, if any, the lymphoid follicles, were in reactive state and capsules were thickened by fibrosis. In what could be identified as early lesions, the centre of the lesions were composed of disintegrating macrophages and a few PMN, with very little or no the pink-staining necrotic material, surrounded by a zone of intact macrophages and PMN. This was followed by a zone of epithelioid cells and a wide zone of macrophages, lymphocytes and few plasma cells. The capsule was not distinct (Fig.4).

Evidence of resolving lesions was observed. Such lesions had no capsule and consisted of a granulation tissue composed of a few lymphocytes, macrophages, angioblasts, immature fibroblasts and sprouts of new capillary blood vessels (Fig.5).

In most lesions, evidence of calcification was apparent as light-blue staining material within the central necrotic area in the H&E stain, and black colouration in Von Kossa stained ssections (Fig.6). One lesion from a sheep had the pus arranged in concentric circles but none of those from goats had such a morphology.

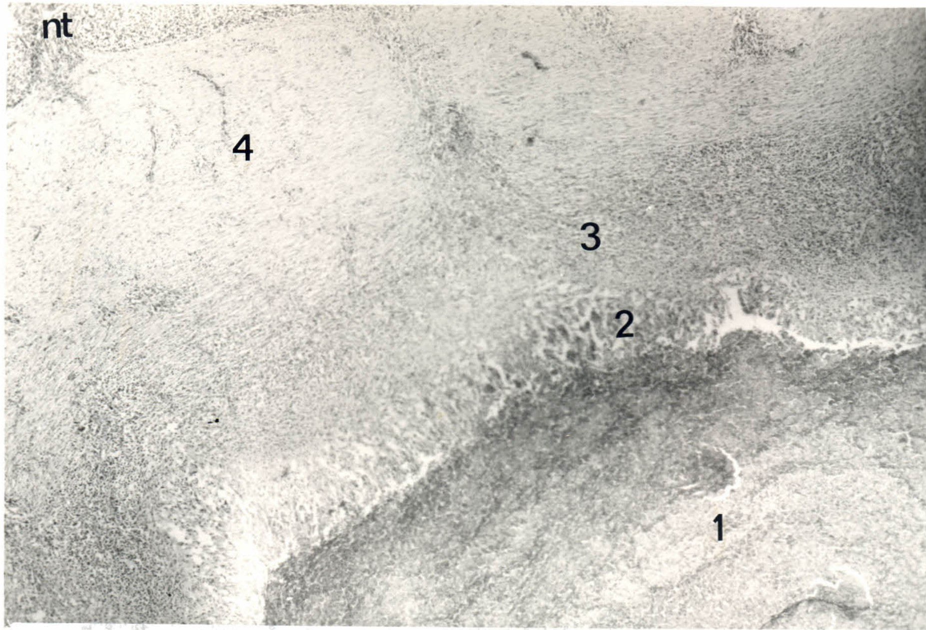


Fig.1

A natural caseous lymphadenitis lesion in a goat showing the necrotic centre (1) surrounded by a zone of epitheliod and giant cells (2), macrophages, lymphocytes and plasma cells (3) and a peripheral layer of fibrous capsule (4). Note normal tissue (nt) at the top left side. H & E x 100.

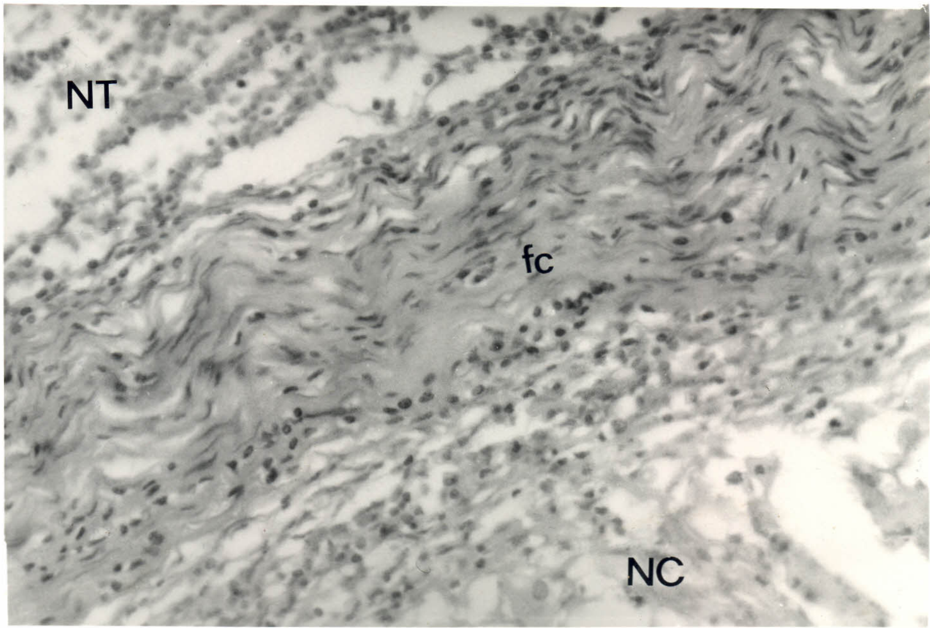


Fig.2

A dense fibrous tissue capsule (fc) in a natural caseous lymphadenitis lesion in a goat. Note the necrotic centre (NC), and normal tissue (NT). H & E x 400.

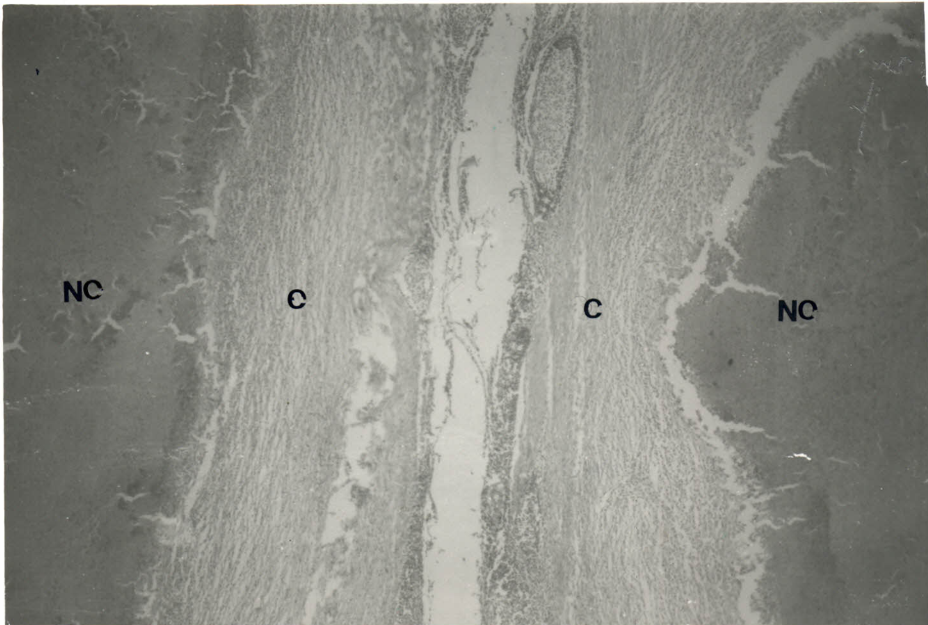


Fig.3

Two caseous lymphadenitis lesions (NC) in a lymphnode of a naturally infected goat. The two lesions are separated by individual fibrous capsules (C). H & E x 100.

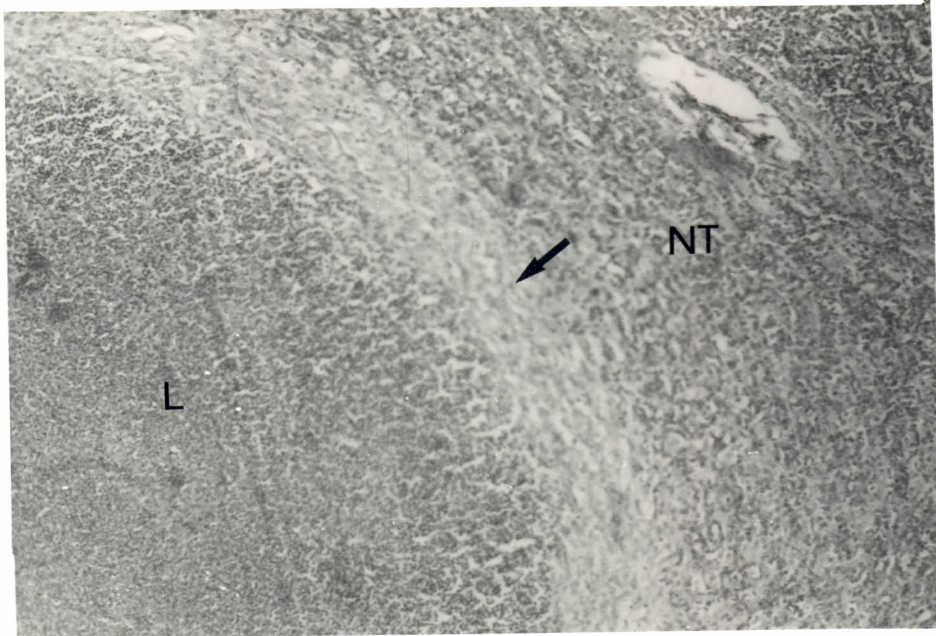


Fig.4

Early caseous lymphadenitis lesion (L) in a naturally infected goat showing the abscence of a capsule. The whitish zone (arrowed) is composed of macrophages, epitheliod and giant cells. Note normal tissue (NT). H & E x 100.

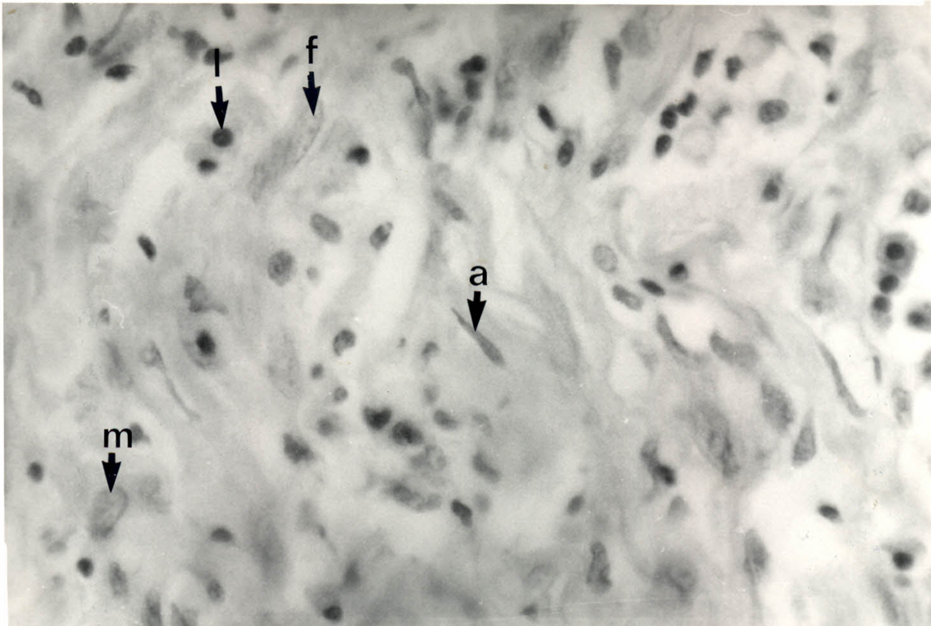


Fig.5

Resolution of a natural caseous lymphadenitis lesion in a goat by formation of granulation tissue composed of macrophages (m) lymphocytes (l) fibroblasts (f) and angioblasts (a). H & E x 630.

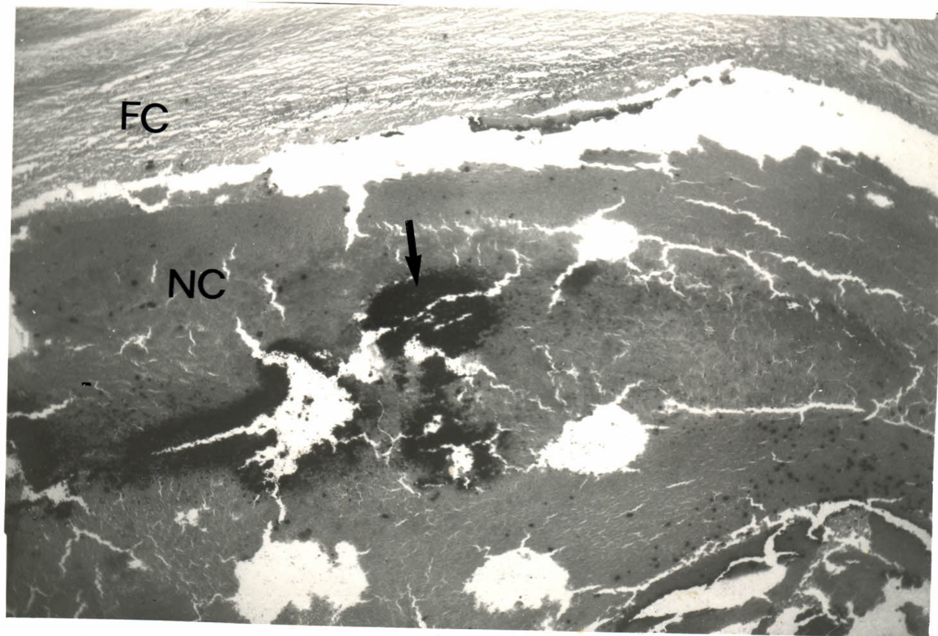


Fig.6

Calcification of a caseous lymphadenitis lesion in a naturally infected goat. The calcified areas are black (arrowed) within the necrotic centre (NC). Note fibrous capsule (FC). Von Kossa x 40.

Lesions adjacent to lymphnodes but with no involvement of the lymphnode had similar morphology to those in lymphnodes. The adjacent lymphnode had reacted by having a thickened capsule and reactive lymphoid follicles.

4.1.3. Bacterial culture.

Bacterial analysis was performed on all the 63 abscessed tissues collected from goats (56) and sheep (7) and the results are summarised in Table 4. *C. pseudotuberculosis* was the most frequently isolated organism, having been isolated from 50 of 63 (79.4%) abscesses. Out of the 50 cases, 36 were pure cultures of *C. pseudotuberculosis* while in 14 cases, *C. pseudotuberculosis* was isolated together with other microorganisms (*Bacillus spp* 7/17 (41%), *Streptococcus spp* 3/17 (18%), *Staphylococcus spp* (5%), *Pseudomonas spp* 3/17 (18%), and coliforms 18%).

Of the abscessed tissues from which *C. pseudotuberculosis* was not isolated (13) seven (11.1%) yielded other microorganisms either in pure or in mixed cultures (*Bacillus* spp 9 cases (14.1 %), *Streptococcus* spp 6 cases (9.5 %), *Staphylococcus* spp 4 cases (6.3 %), *Pseudomonas* spp 1 case (1.6 %) and coliforms 4 cases (9.5 %), while another 6 (9.52%), including the lung abscess from a sheep were found to be sterile (Table 4).

Table 4.

The type and frequency of isolation of microoganisms from sheep and goat caseous abscesses collected from natural pseudotuberculosis lesions.

Organism.	Culture type. Pure.	Mixed.	Total.
C. Pseudotuberculosis	36	14	50
Bacillus spp	1	8	9
Streptococcus spp	2	4	6
Staphylococcus spp	3	1	4
Coliforms	0	4	4
Pseudomonas spp	0	1	1
None	-	-	6

4.1.4. Serological findings.

Two hundred seventy three serum samples were collected from 57 carcasses with and 216 carcasses without pseudotuberculosis lesions, respectively. BAT titres for samples from animals without lesions ranged from 0.9 to 3.0. The most frequent titre was 1.8 (46.75%). Titres for samples from animals with lesions ranged from 1.2 to 4.2 (Fig.7a). The most frequent titre was 2.1 (28%). Titres for samples from animals with lesions from which *C. Pseudotuberculosis* was isolated ranged from 1.5 to 4.2 (Fig.7a). The most frequent titre was 2.1 (24.4%). There was no significant difference in the percent distribution of the various titres between samples from animals with lesions and those from animals with lesions from which *C.Pseudotuberculosis* was isolated ($P=0.025 < 0.05$). Statistical difference in titre distribution between animals with lesions and those without was significant ($p=0.0001 < 0.05$).

HIT titres for samples from animals without lesions ranged from less than 0.3 to 1.8 (Fig.7b), the most frequent titre being less than 0.3 (91.20%). Titres for samples from animals with lesions ranged from less 0.3 to 2.7. The most frequent titre was 0.6 (20.7%). Samples from animals with lesions from which *C. pseudotuberculosis* was isolated had titres ranging from less than 0.3 to 2.7 and the most frequent titre was 0.9. (21.4%). There was no significant difference in the percent observation of the various titre values between samples from animals with lesions and those from animals with lesions from which *C. pseudotuberculosis* was isolated ($p= 0.025 < 0.05$). Titre distribution difference between animals with lesions and those without was statisticaly significant ($p=0.0001 < 0.05$).

Table 5 shows the postmortem, bacteriological and serological examination results from the 57 animals with and the 216 without lesions. Of those with lesions, 50 had positive BAT titres, 46 were positive for HIT and 51 were positive for either test. The sensitivities

were calculated as 87.7% for BAT, 80.7% for HIT and 89.47% for the combined tests. Considering only the animals from which *C. pseudotuberculosis* was isolated, the sensitivities were calculated as 93.3% for BAT, 88.88% for HIT and 95.5% for the combined,(either) tests.

Twenty-six of the 216 animals (12%), with no lesions were positive for BAT, 6 for HIT and 28 for either test. The specificities were calculated as 87.96 for BAT, 97.22% for HIT and 87.03 for the combined tests.

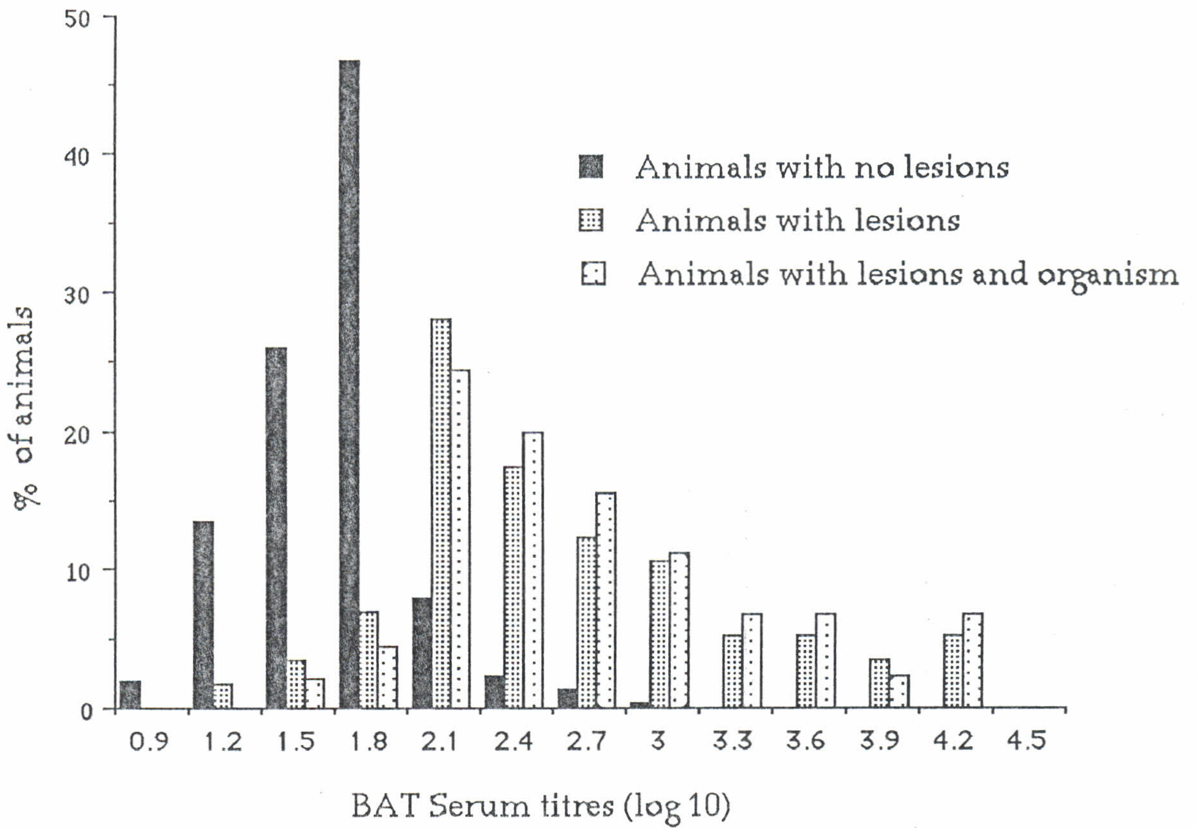


Fig.7a

Distribution of Bacterial agglutination test (BAT) titre values among sheep and goats with and without caseous lymphadenitis during postmortem inspection.

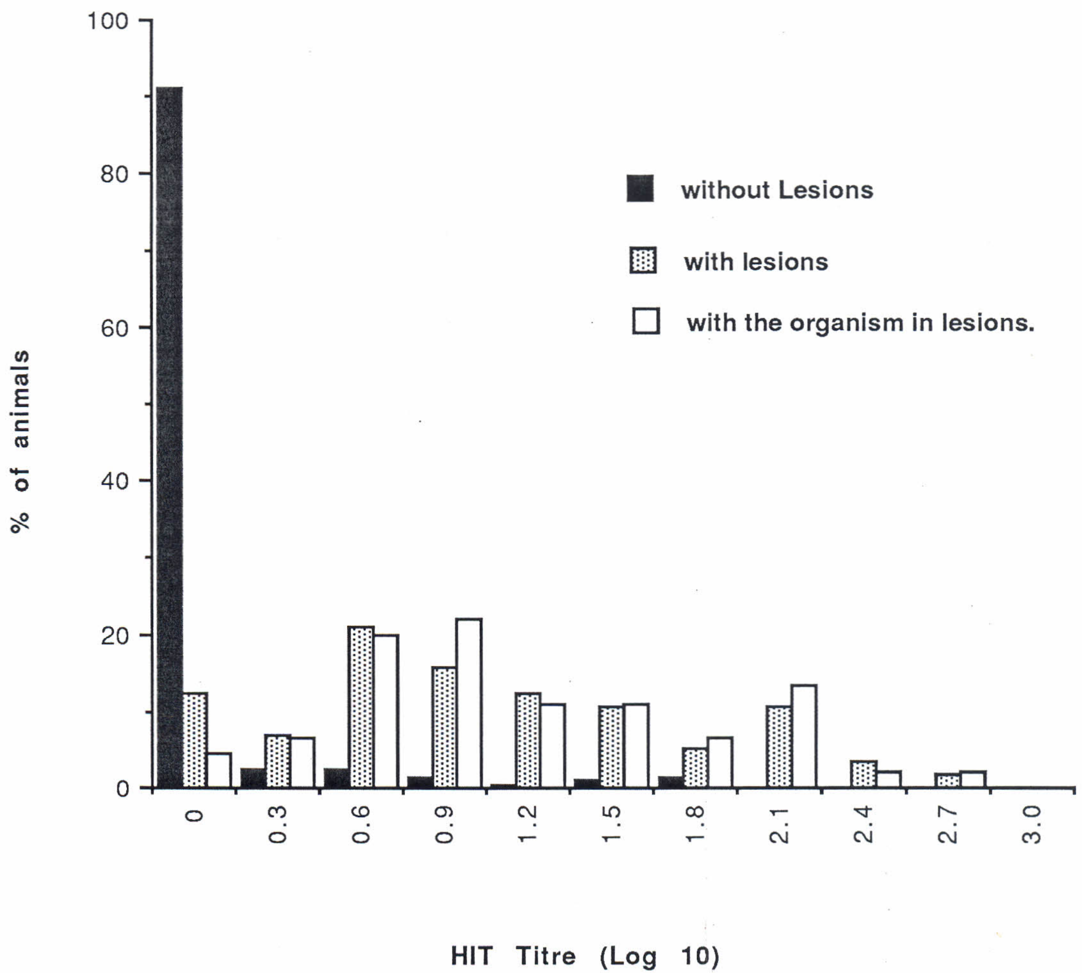


Figure 7b. Distribution of Haemolysis Inhibition test (HIT) titres, among sheep and goats with or without caseous lymphadenitis during postmortem meat inspection.

Table 5: Bacterial agglutination test (BAT) and Haemolysis inhibition test (HIT) results of sera from sheep and goat carcasses with and without caseous lymphadenitis during postmortem inspection and the correcponding bacteriological findings.

Postmortem findings	Bacteriological Results	BAT Positive	HIT Positive	BAT and HIT		BAT or HIT Positive
				Positive	Negative	
A) Lesions present	i) *C.pseudotuberculosis*	42/45	40/45	39/45	2/45	43/45
	ii) Other orgganisms	6/8	5/8	5/8	2/8	6/8
	iii) Sterile	2/4	1/4	1/4	2/4	2/4
	Total	50/57	46/57	45/57	6/57	51/57
B) No lesions	-	26/216	6/216	4/216	188/216	28/216

4.2. The routes of infection.

4.2.1. Clinical examination.

Results of clinical examination of test lymphnodes over a nine week period are given in Table 6. In group 1 (innoculated subcutaneously), goat number 12 died of pneumonia three days post infection. On day two, the two remaining animals in the group (Nos. 1 and 2) were lame on the right hind leg. Injection sites were tender. The highest recorded rectal temperatures were 39.8°C and 38.5°C respectively. On day three, the animals were weak, inappetent and injection sites were swollen. Lameness was not apparent after the first week. On the third week, swellings of the injection sites were hard and sharply defined while the prescapular site in Goat No. 1 was discharging creamy odourless pus. The test precrural node of Goat No. 1 and both prescapular and precrural nodes of Goat No. 2 were palpably swollen. By the fourth week, all injection sites were discharging and test precrural nodes in both animals were visibly swollen.

In group 2 (innoculated intradermally), injection sites and test lymphnodes were swollen by day three in all three goats. Rectal temperatures were 38.0°C, 37.7°C and 37.7°C, respectively. Test lymphnodes were swollen by day four and by day six, injection sites were discharging pus through fistulae. Two weeks post infection, there was caked pus on all injection sites. This was followed by scab formation, sloughing off and healing of the skin by week four. Goat No. 5 died of pneumonia on day 21. Goat No. 6 developed a subcutaneous abscess close to the test prescapular lymphnode.

In group 3 (infected on scarified skin), animals infection sites were slightly swollen by day one all three animals. Rectal temperatures were 37.2°C, 37.4°C and 37.5°C respectively. Test lymphnodes in Goat No.8 were swollen by day three. After week one, there was scab formation followed by healing of infection sites in all three animals. Test

lymphnodes in goats Nos. 10 and 11 were slightly swollen by week two. Goat number eight died of pneumonia on day 36. Test precrural node in goat number 11 was visibly swollen by week five.

In groups 4 (infected on intact skin) and 5 (control), Goat No. 9 died of a non-determined cause five days after infection. There was no clinically detectable changes in the rest of the animals in the two groups during the cause of the experiment.

4.2.2. Haematological findings.

White blood cell counts increased in groups 1, 2 and 3 up to the second week post infection and then dropped back to the original level by week six. The highest increase was recorded in group 1 followed by 2 and 3 respectively (Fig.8). No change in counts was observed in groups 4 and 5.

Statistical analysis performed by a one factor (group) analysis of variance indicated that the variation of means between groups was significant ($p=0.0001$). The Scheffe's F-test indicated that on the basis of means, the groups could be separated into three namely 1 and 2; 2,3 and 4; and 5 alone (Table 7).

Table 6: Clinical and postmortem (pm) findings in regional lymphnodes of goats infected with ***C. pseudotuberculosis*** through various routes.

CLINICAL SWELLING

GROUP	INFECTION ROUTE	GOAT NO.	LYMPHNODE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 9	ABSCESS AT PM
1	S/C	1	A	-	-	-	-	-	-	-	-	-	-	-	+
			B	-	-	-	-	-	-	-	-	+	+	+	++
		2	A&B	-	-	-	-	-	-	-	-	+	++	++	++
		12	A&B	0	0	0	0	0	0	0	0	0	0	0	-
11	1/D	5	A&B	-	-	+	+	+	+	+	+	+	0	0	++
		6	A&B	-	-	+	+	+	+	+	+	+	++	++	++
		7	A&B	-	-	+	+	+	+	+	+	+	+	+	+
111	Scarification	8	A&B	-	-	+	+	+	+	+	+	+	+	0	+
		10	A&B	-	-	-	-	-	-	-	+	+	+	+	+
		11	A&B	-	-	-	-	-	-	-	+	+	+	++	+
IV	Intact skin	13	A&B	-	-	-	-	-	-	-	-	-	-	-	-
		14	A&B	-	-	-	-	-	-	-	-	-	-	-	-
		9	A&B	0	0	0	0	0	0	0	0	0	0	0	-
V	Control	3	A&B	-	-	-	-	-	-	-	-	-	-	-	-
		4	A&B	-	-	-	-	-	-	-	-	-	-	-	-

KEY:

Lymphnode A = prescapular; B = precrural; + = positive
i/d = intradermal; - = Negative; 0 = No data.
++ = Markedly positive; s/c = Subcutaneous;

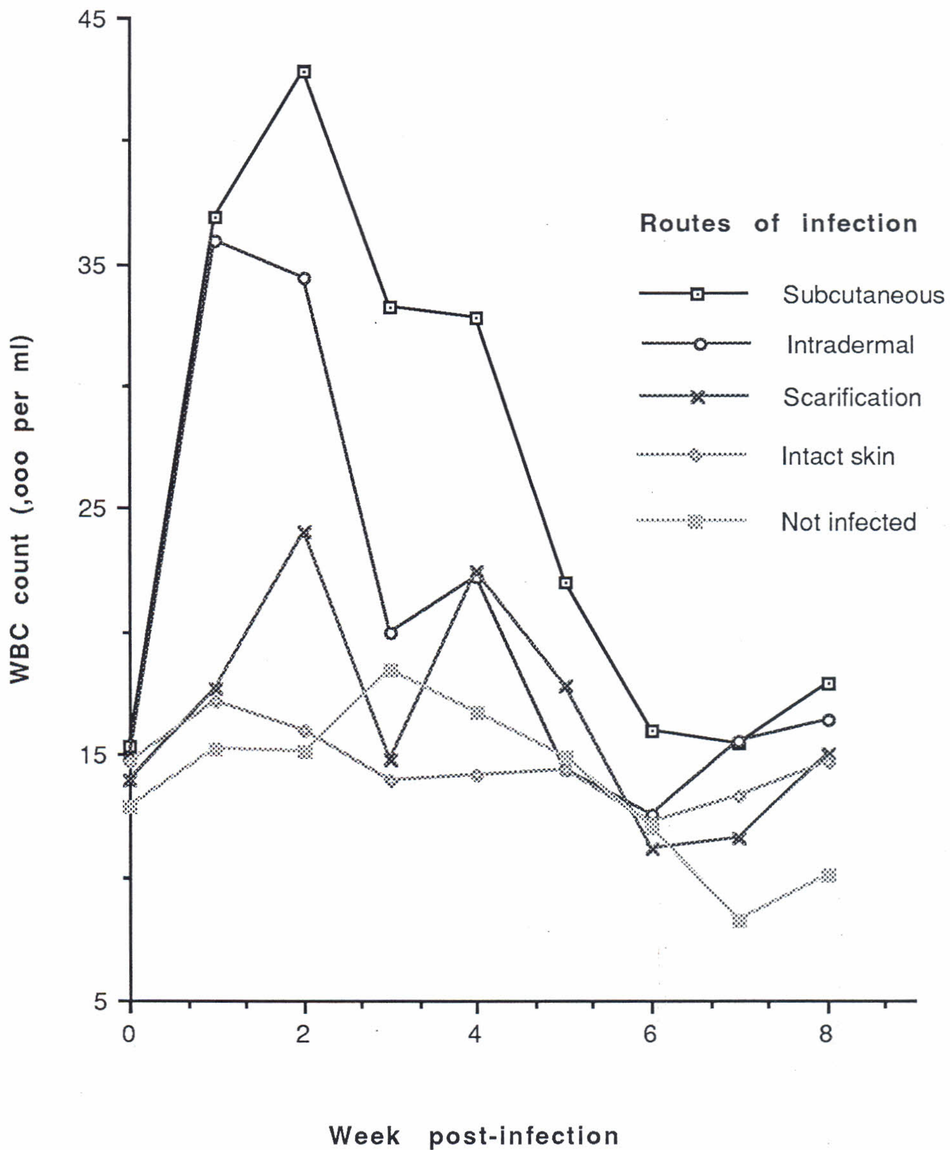


Figure 8. Changes in white blood cell (WBC) counts in goats following infection with C. pseudotuberculosis through different routes.

The percent blood neutrophil count also increased, and more significantly in the first three groups, an indication that the increase in white blood cell count was partly due to a neutrophilia. Figure 9 shows the group average weekly % neutrophil count. The variance between the groups was significant (P=0.0001) with significant Scheffe's F-test differences between the means in groups 1 and 4, 1 and 5, 2 and 4, and 2 and 5 (Table 7).

The total plasma protein values increased in all groups during week one and then decreased to below the original concentrations (Fig.10). The variance between the groups was significant (P=0.0001) and the groups could be separated into two on the basis of the means namely 1, 2, 3 and 4 together and 5 separately (Table 7).

The values of the haematocrit (PCV) decreased over the experimental period in all groups including the control group. The decrease was most noticeable in groups 2, 3 and 5 (control) and least in group 4. The Haemoglobin concentration decreased in a similar pattern as the PCV. There was no significant variance in PCV and haemoglobin values between the groups ($P=0.0869 > 0.05$)

Table 7.

Scheffe's F-test for separation of mean haematological values in goats infected with *C. pseudotuberculosis* through different routes.

Haematological parameter.	**Route of infection.**				
	1	2	3	4	5
Leucocyte count	25.8^{a}	21.7^{ab}	17.0^{abc}	14.7^{bc}	13.7^{c}
% Neutrophil count	1.4^{a}	1.3^{a}	0.9^{ab}	0.7^{b}	0.5^{b}
Haematocrit	26.7^{a}	25.7^{a}	26.3^{a}	24.6^{a}	23.9^{a}
Total plasma protein	6.70^{a}	7.3^{a}	6.9^{a}	6.7^{a}	5.9^{b}

Key: Route 1 = Subcutaneous

2 = Intradermal

3 = Scarified skin

4 = Intact skin

5 = Not infected

Means with different superscripts are significantly different within rows ($p < 0.05$).

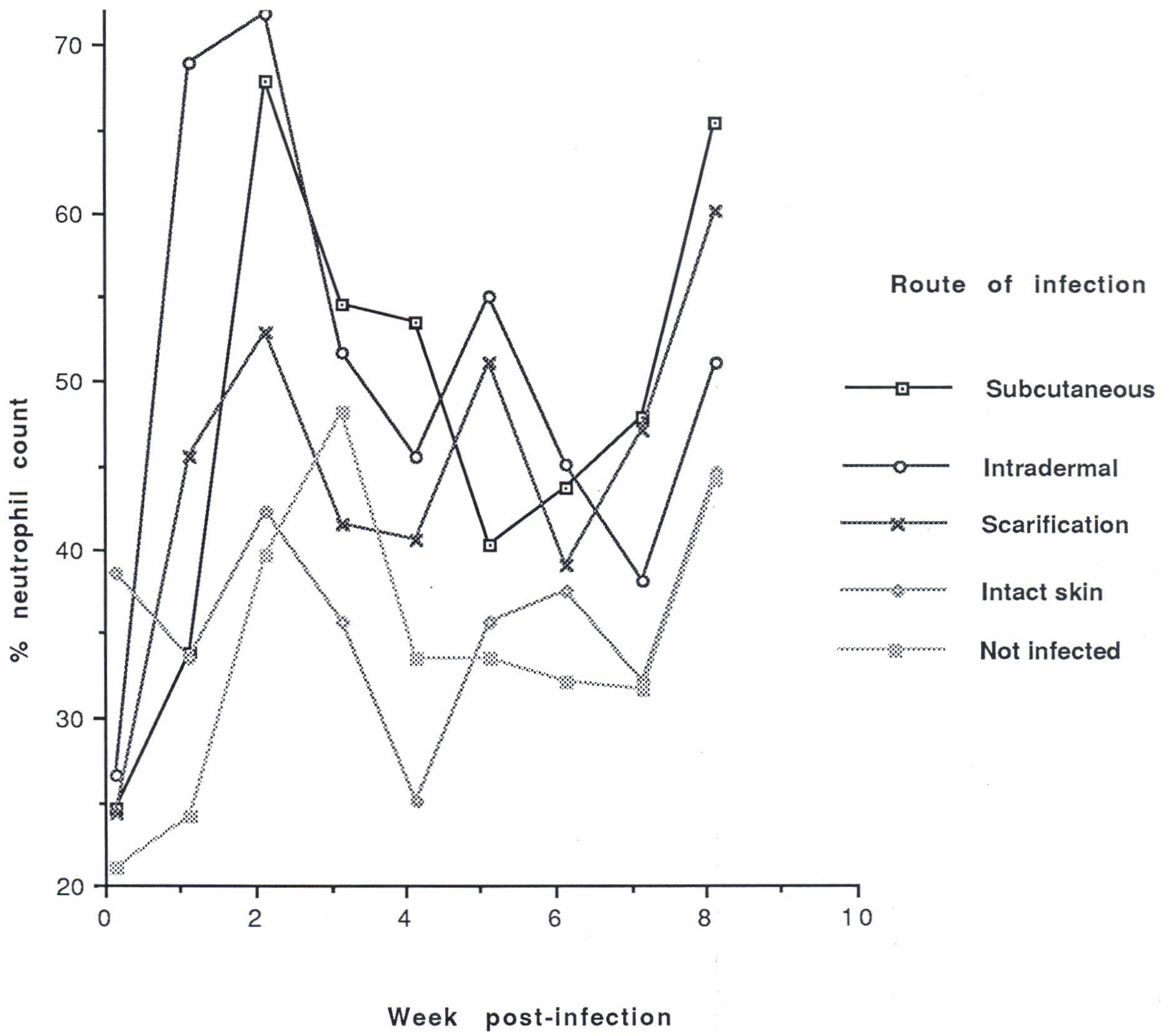


Figure 9. Changes in % neutrophil count in goats following exposure to C. pseudotuberculosis through different routes.

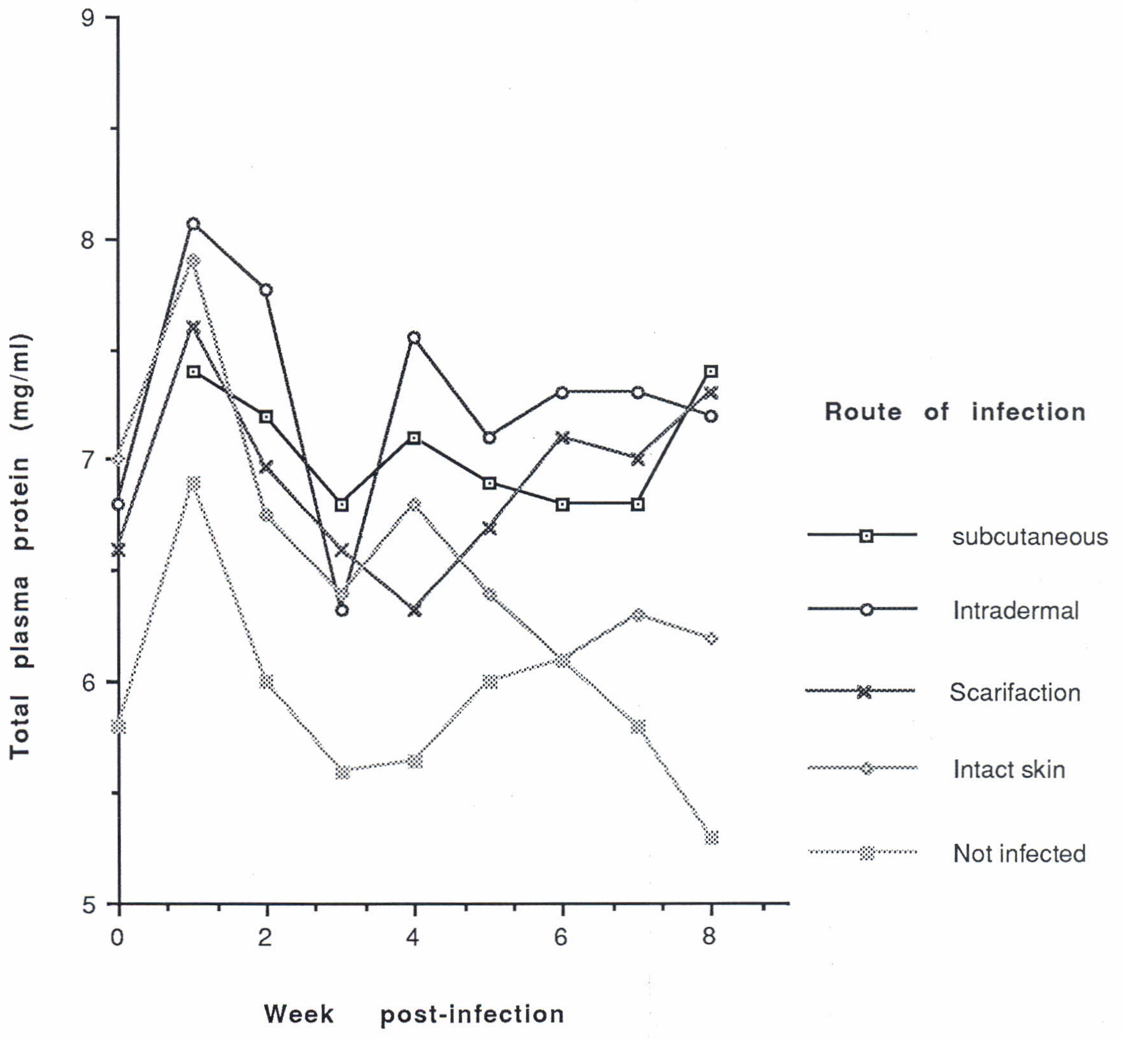


Figure 10. Changes in total plasma protein concentration in goats following infection with *C. pseudotuberculosis* through different routes

4.2.3. Postmortem findings.

Test prescapular and precrural lympnodes of animals in groups 1,2 and 3 were abscessed and enlarged (Figs. 11-14). The results are summarised in Table 6. No other lymphnodes, superficial or internal, or organ was found abscessed. Group 1 animals also had subcutaneous abscesses at the sites of injection and Goat No.6 of group 2 had a subcutaneous abscess close to the test prescapular node.

C.pseudotuberculosis was isolated from all abscesses in pure cultures. Test lymphnodes in groups 1, 2 and 3 were found to weigh more than the control lymphnodes. Figure 15 shows the mean comparative weights. The difference in weights between test and control nodes was most marked in groups 1 and 2. It was negative for precrural nodes in group 4 and for both lymphnodes in the control group. The weight differences between test and control lymphnodes was statistically significant, ($P=0.02 < 0.05$) for both precrural and prescapular lymphnodes. Statistical difference in lymphnode weights between groups (routes of infection) was significant for precrural lymphnodes ($P=0.04 < 0.05$) but not for prescapular lymphnodes ($P=0.08 > 0.05$).

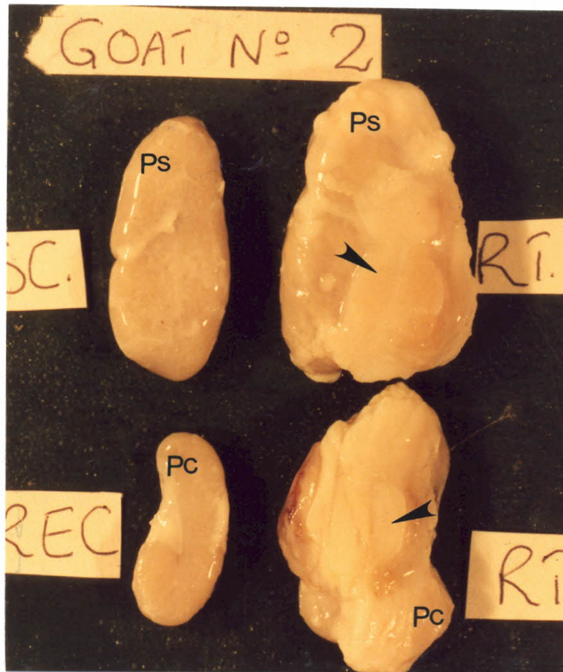


Fig.11

Prescapular (ps) and precrural (pc) draining lymphnodes in a goat infected subcutaneously with *C. pseudotuberculosis* caseous abscess material, showing enlargement and abscessation of the test lymphnodes (right) as compared to the contralateral control (left) lymphnodes. Note the creamy caseous material in the test lymphnodes (arrows).

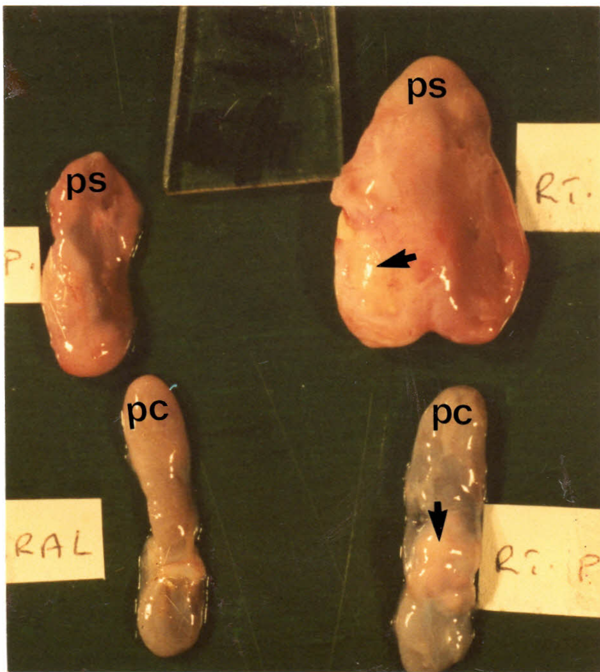


Fig.12

Draining lymphnodes, prescapular (ps) and precrural (pc) in a goat infected intradermally with *C. pseudotuberculosis* caseous abscess material, showing enlargement and abscessation of the test lymphnodes (right) as compared to the contralateral control (left) lymphnodes. Note the large protruding caseous abscess (arrows) on the test lymphnodes.

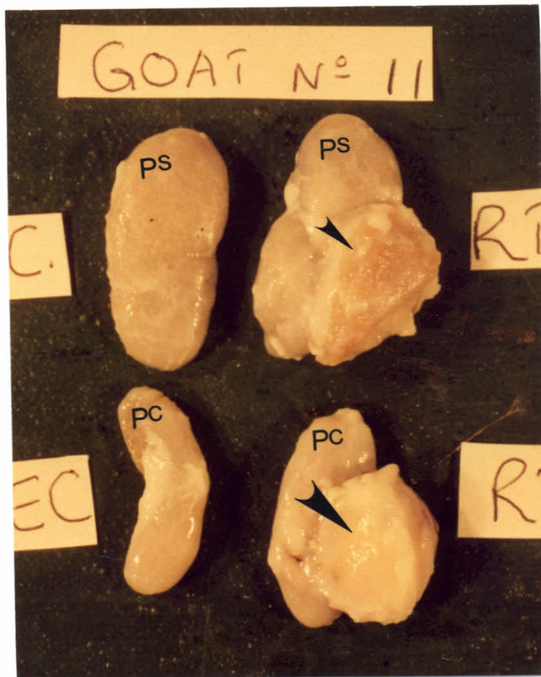
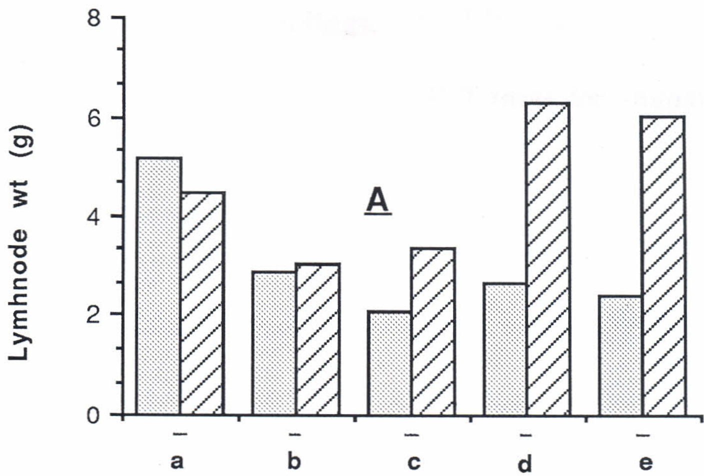


Fig. 13.

Prescapular (ps) and precrural (pc) lymphnodes in a goat infected on scarified skin with *C. pseudotuberculosis* caseuos abscess material, showing enlargement and abscessation of the test lymphnodes (right) as compared to the contralateral (control) lymphnodes on the left. Note the marked enlargement of the right prescapular lymphnode and the caseous abscesses (arrows).

Fig.14

Draining lymphnodes, prescapular (ps) and precrural (pc) in a goat infected on intact skin with *C. pseudotuberculosis* caseous abscess material showing failure of abscessation of both the test (right) as well as the contralateral control (left) lymphnodes.



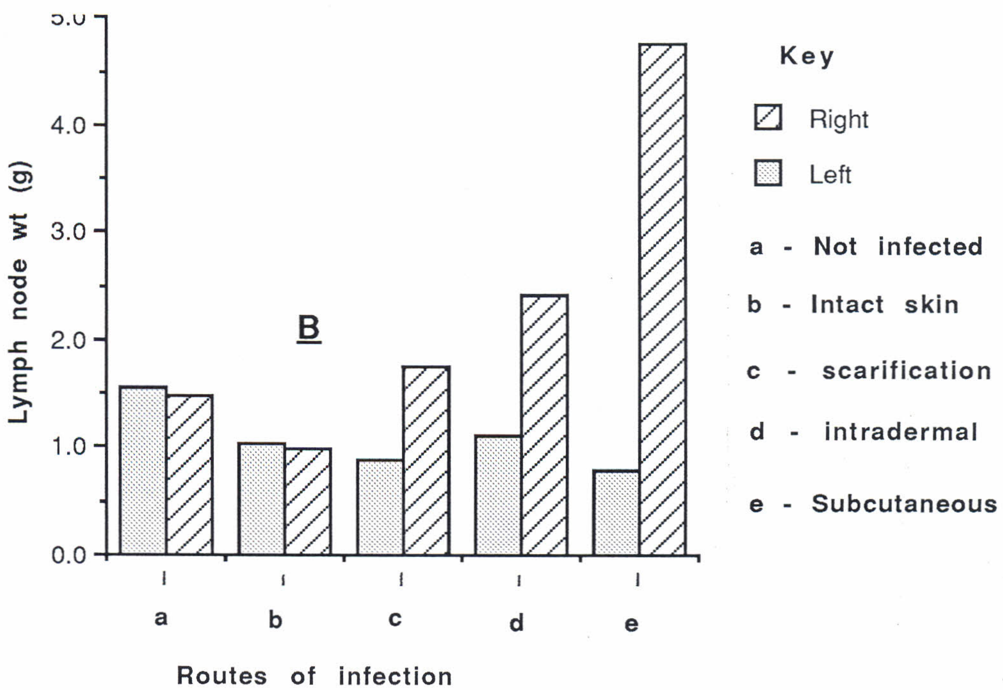


Figure 15. Weights of draining lymph nodes (prescapular (A) and precrural (B)) in goats infected with *C. pseudotuberculosis* through various routes.

4.2.4. Serological findings.

Weekly mean BAT and HIT titres for animals infected through the different routes are shown in Figure 16a and 16b, respectively. Titres were positive for animals in group 1 and 2 by week two except for Goat Nos.1 and 5 which were not HIT positive until week four and three respectively. In group 3, BAT titres were positive by week two (except Goat No.10) and HIT positive by week three. In both tests, the highest titres were observed in group 2 (Intradermal infection) followed by group 1, (Subcutaneous infection) and 3 (Scarified skin), respectively.

A two factor analysis of variance comprising of group (route of infection) and week post infection (time) showed that the mean titre differences between groups was significant (P=0.001) indicating that some groups attained higher titres and at a faster rate than others. The difference in the weekly increase in titres was also significant (P=0.0001) indicating that some groups responded faster than others.

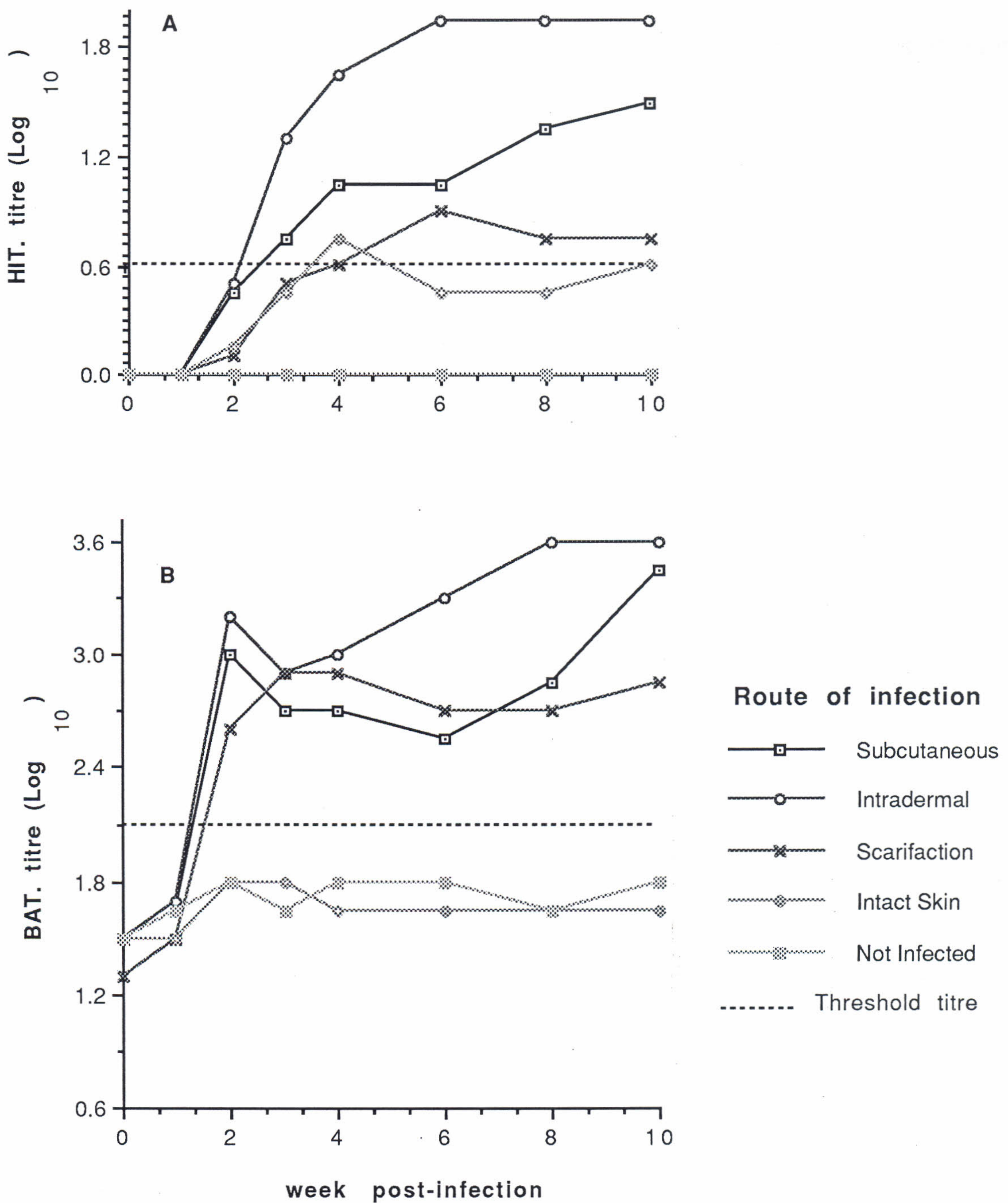


Figure 16. Serological response in goats following infection with C. pseudotuberculosis through different routes. A=Haemolysis inhibition test (HIT). B=Bacterial agglutination test (BAT).

4.3. The minimum dose of infection for CLA.

4.3.1. Clinical examination findings.

A summary of clinical and postmortem findings in test lymphnodes is given in Table 8. Test lympnodes of animals in group 1 were palpably swollen by day two. Injection sites were also swollen and tender. The animals had a slight pyrexia of 38.5°C and 38.2°C, respectively. Animals in group 2 had slightly swollen injection sites by day two and test lymphnodes were palpably swollen by day four. There was no swelling or inflamation of injection sites in groups 3-8 but lympnode enlargement was detected in some or all animals in group 3 on day six; in groups 4 and 5 on day 21; in group 6 on day 28; and in group 7 on day 21. There was no palpable enlargement of lymphnodes detected in group 8.

4.3.2. Haematological findings.

The values of white blood cell count (WBC) the haematocrit (PCV), the haemoglobin concentration (HB) and the total plasma protein (TP) were significantly different between the eight groups (P=0.0005 for WBC; 0.0016 for PCV; 0.007 for HB; and 0.0376 for TP). However, the changes in these parameters were haphazard. As such, separation of means by a Scheffe's F- test was not possible.

Table 8: Clinical and postmortem findings in draining lymphnodes of goats infected intradermally with different doses of *C. pseudotuberculosis*.

GROUP	GOAT NO.	LYMPH NODE	Clinical swelling/Days post infection 1	2	3	4	5	6	7	9	11	21	28	35	PM	TOTAL
1	15	A&B	-	+	+	+	+	+	+	+	+	+	+	+	CL	4
	16	A&B	-	+	+	+	+	+	+	+	+	+	+	+	CL	
2	17	A	-	±	±	+	+	+	+	+	+	+	+	+	NCL	3
	22	B	-	±	±	+	+	+	+	+	+	+	+	+	CL	
		A&B	-	±	±	+	+	+	+	+	+	+	+	+	CL	
3	18	A&B	-	-	-	-	±	+	+	+	+	+	+	+	CL	4
	19	A&B	-	-	-	-	-	±	±	±	±	+	+	+	CL	
4	27	A	-	-	-	-	-	-	-	-	-	-	-	-	CL	3
	21	B	-	-	-	-	-	±	±	±	±	±	±	±	CL	
		A	-	-	-	-	-	-	-	-	-	-	-	-	CL	
		B	-	-	-	-	-	-	-	-	-	+	+	+	CL	
5	29	A&B	-	-	-	-	-	-	-	-	-	+	+	+	CL	3
	30	A	-	-	-	-	-	-	-	±	±	+	+	+	CL	
		B	-	-	-	-	-	-	-	-	-	-	-	-	NCL	
6	23	A	-	-	-	-	-	-	-	-	-	-	+	+	CL	2
	24	B	-	-	-	-	-	-	-	-	-	-	-	-	NCL	
		A	-	-	-	-	-	-	-	-	-	-	+	+	CL	
		B	-	-	-	-	-	-	-	-	-	-	-	-	NCL	
7	31	A	-	-	-	-	-	-	-	-	-	-	-	-	NCL	1
	32	B	-	-	-	-	-	-	-	-	-	+	+	+	CL	
		A&B	-	-	-	-	-	-	-	-	-	-	-	-	NCL	
8	25	A&B	-	-	-	-	-	-	-	-	-	-	-	-	NCL	0
	26	A&B	-	-	-	-	-	-	-	-	-	-	-	-	NCL	

KEY:
A = Prescapular lymphnode
B = Precrural lymphnode
- = Not swollen
+ = Swollen
± = Swelling not definate
CL = Abscessed
NCL = Not abscessed
PM = Postmortem

4.3.3. Postmortem Findings.

All four (4/4) test lympnodes of animals in group 1 were found abscessed at postmortem (Table 8). The frequency of abscessation was as follows: 3/4 in group 2, 4/4 in group 3, 3/4 in group 4, 3/4 in group 5, 2/4 in group 6, 1/4 in group 7 and 0/4 in group 8. The average difference in weight between the test and control lymphnodes in each group is shown in Fig. 17. Twelve out of sixteen (12/16) prescapular and 13/16 precrural test lymphnodes weighed more than the contralateral control lymphnodes. The difference in weights within groups was statistically significant by an unpaired students T-test ($P=0.002$ for precrural and $P=0.04$ for prescapular lymphnodes, respectively). The difference in weights between test and control lymphnodes was not statistically significant between the groups ($P= 0.10$ for precrural and 0.17 for prescapular lymphnodes respectively). However, with the exception of groups 5 and 6, the decrease of the difference with decrease in dose of infection was quite apparent (Fig.17).

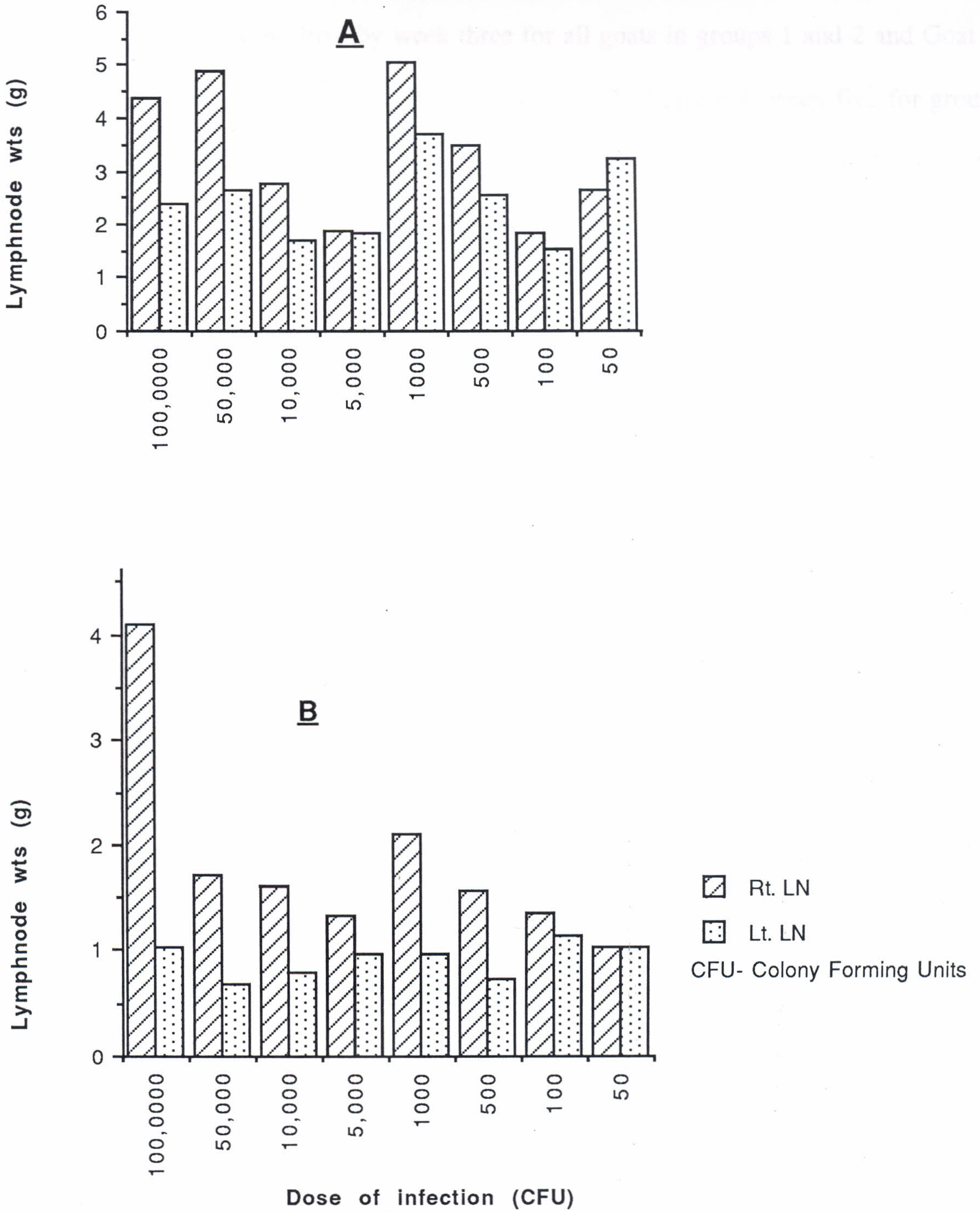


Figure 17. Weights at postmortem of draining lymphnodes (prescapular, (A) and precrural, (B) in goats following intradermal infection with different doses of C. pseudotuberculosis.

4.3.4. Serological findings.

HIT titres were positive by week three for all goats in groups 1 and 2 and Goat No. 21 of group 4, week four for group 3 and Goat No. 27 of group 4, week five for groups 5 and 6, and after week six for groups 7 and 8 with the exception of Goat No.32 and 26 from which no positive titres were observed (Fig.18A).

BAT titres were positive for all goats in groups 1, 2,3 and 4 by week two post infection (Fig. 18B) except for Goat No. 27 of group 4 which was not positive until week three. In group 5, Goat No. 30 remained negative throughout the experimental period while Goat No. 29 had positive titres from week four onwards. In group 6, Goat No. 23 was positive from week six while Goat No. 24 had marginally positive titres (2.1) between weeks three and four only. In group 7 and 8, positive titres were observed only in No. 32, from week three onwards, while the rest, including No. 31 which had an abscessed lympnode at postmortem, remained negative throughout.

A two factor analysis (group versus week) of variance indicated that mean titres differences between groups were statistically significant (P = 0.001 for both tests) and that the difference in the weekly titre changes between groups were
significant (P=0.001). The mean titres were higher and also rose faster with higher doses of infection.

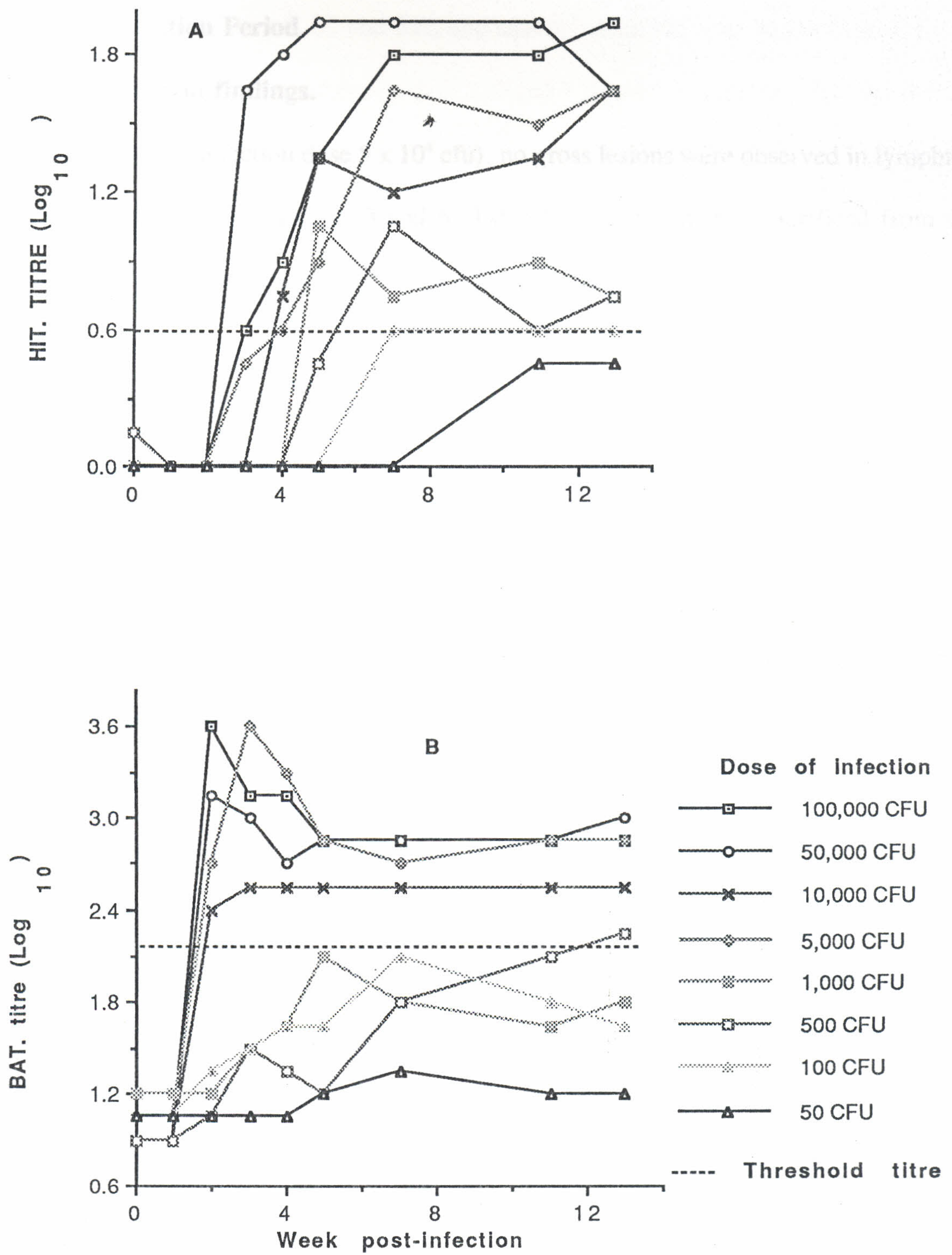


Figure 18. Serological response in goats following intradermal infection with different doses of *C. pseudotuberculosis*. A=Haemolysis inhibition test (HIT). B=Bacterial agglution test (BAT).

4.4. Incubation Period.

4.4.1. Postmortem findings.

In group 1 (infection dose 5 x 10^4 cfu), no gross lesions were observed in lymphnodes of animals sacrificed at days 2, 3 and 6 (Table 9), but all animals sacrificed from day 8 onwards had one or both lymphnodes abscessed. Injection sites of all animals were abscessed except in one (goat No.41) which the sites had healed, at day 34.

Microscopic lesions were observed in sections taken at days 2-6. The lesions were either bands or zones of macrophages and a few PMN infiltration, starting from the cortex and extending towards the medulla (Fig. 19), or focal accumulation of these cells in trabecular sinuses. The infiltrating macrophages had a strongly oesinophilic cytoplasm. There was also oedema of the sinuses (capsular and medullary), inflammation of the capsule and follicular hyperplasia. In some sections there was also fibrin-like oesinophilic deposits in lymphnode follicles and in medullary sinuses.

At days 8 and 11, both lymphnodes had multiple foci of pyogranulomatous lesions of varying sizes and stages of development (Fig. 20). The lesions tended to be confined to one half of the lympnode next to afferent lymph vessels. Some foci had necrotic centres with nuclear debris while others had mononuclear cells, (macophages), and PMN in the process of degeneration. The foci were surrounded by a zone of large macrophages and immature epithelioid cells. Capsules in the affected parts of lymphnodes were thickened by fibrosis and cellular infiltration with capsular sinuses being obliterated. Lympnodes sections at days 17, 25, and 34 had one or more capsulated lesions with typical CLA morphology (Fig. 21).

In the group 2 animals (infected with 1 x 10^5 cfu), abscessed lymhnodes were observed in all goats sacrificed from day nine onwards except in one (goat No.51) sacrificed at day 20. Injection sites abscessed in all goats except in two in which there was only

necrosis at the surface (nos. 50 and 51), and one in which the sites had healed, (No. 42).

Microscopically, the picture at days 2, 5 and 7 showed marked oedema, capsulitis and marked follicular hyperplasia. At days 9, 11, 14 and 17, both lymphnodes of Goats 4Nos. 6, 47 and 50 and the precrural node of Goat No. 48 had multiple focal pyogranulomas of differenct sizes, with or without capsulations. Lesions in goat 48 were surrounded by an extensive zone of lymphoid necrosis while prescapular node of the same animal (no grossly visible lesion), had one microscopic pyogranuloma within a locally extensive are of necrosis. In the unaffected parts of hymphnodes, all four animals had marked follicular hyperplasia.

In goat 51, (sacrificed at day 20) although no grossly visible lesions were found, a microscopic granuloma located adjacent to the capsule and surrounded by a zone of fibrosis was observed in the precrural node.

Table 9: Postmortem findings in skin and draining lymphnode and BAT and HIT titres in goats infected with $5x10^4$ colony forming units (cfu) of *C. pseudotuberculosis* (Group 1), 10^5 cfu (Group 2) or with caseous abscess material on scarified skin (Group 3).

			SITE 1		SITE 2		SEROLOGY	
GROUP	GOAT NO.	DAY SACRIFICED	LN	SKIN	LN	SKIN	BAT	HIT
1	33	2	-	+	-	+	1.2	-
	34	3	-	+	-	+	1.2	-
	35	3	-	+	-	+	1.5	-
	36	6	-	+	-	+	1.5	-
	73	8	+	+	+	+	1.5	-
	38	11	+	+	+	+	1.5	-
	39	17	+	+	-	+	3.0	0.3
	40	25	-	+	+	+	3.0	0.6
	41	34	+	H	+	H	3.0	2.4
2	43	2	-	+	+	+	1.8	-
	49	5	-	+	+	+	1.8	-
	44	7	-	+	+	+	1.8	-
	46	9	+	+	+	+	1.8	-
	47	11	+	+	+	+	1.5	-
	48	14	-	+	+	+	1.8	-
	50	17	+	Nec.	+	Nec.	2.7	-
	51	20	-	Nec.	-	Nec.	3.6	1.2
	52	27	+	+	+	+	3.6	0.6
	42	30	+	H	+	H	2.7	0.9
3	62	5	-	-	-	-	1.2	-
	67	7	-	-	-	-	1.2	-
	70	9	-	-	+	-	0.9	-
	68	11	+	-	+	-	1.2	-
	175	13	+	-	+	-	1.5	-
	71	15	+	-	-	-	2.1	-
	64	17	+	-	+	-	2.1	-
	66	19	+	-	+	-	2.4	-
	69	21	+	-	-	-	2.7	0.9
	72	23	+	-	+	-	2.4	1.2

KEY:

+	= Abscessed	H	= Healed
-	= Not abscessed	Site 1	= Prescapular
Nec.	= Necrosis	Site 2	= Precrural

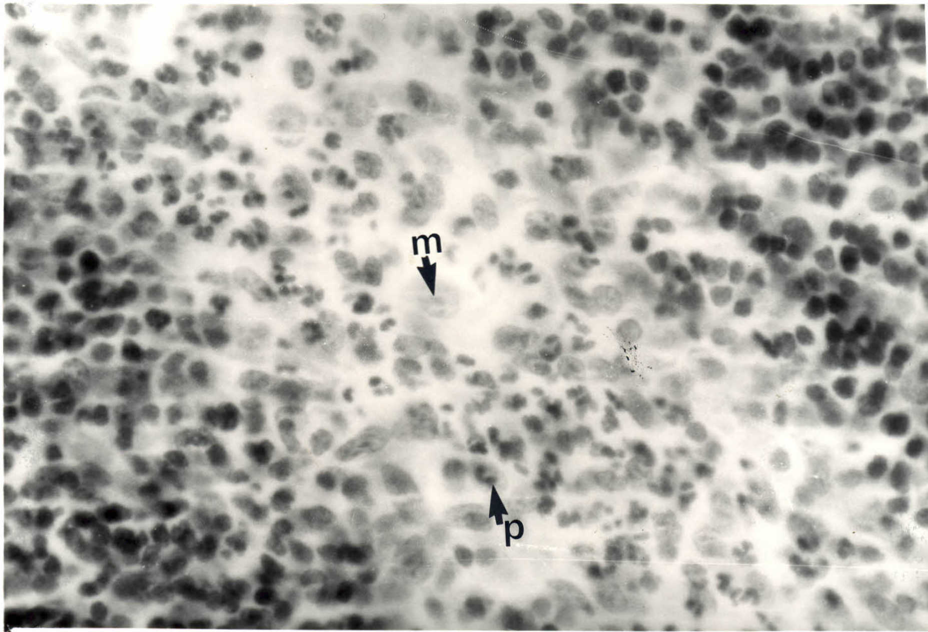


Fig.19

The initial lesion in a goat prescapular lymphnode showing a band composed of macrophages (m) and polymorphonuclear granulocytes (p) infiltration 2 days after intradermal infection with 5 x 10^4 cfu of *C. pseudotuberculosis*. H & E. x 630.

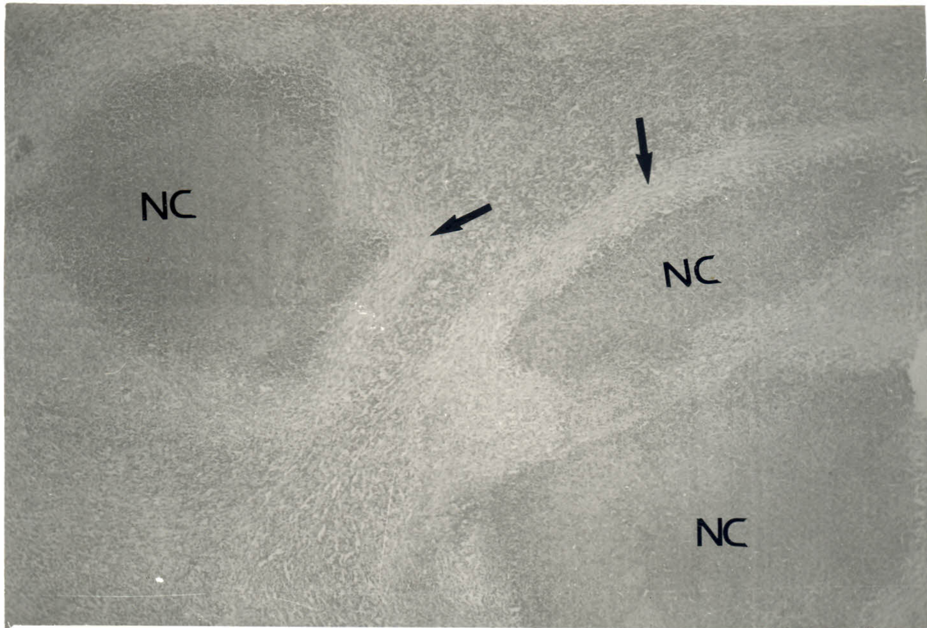


Fig.20

Multiple caseous lymphadenitis lesions in the prescapular lymphnode of a goat, 11 days after intradermal infection with 5 x 10^4 cfu of *C. pseudotuberculosis*. Note the whitish zone (arrowed) surrounding each necrotic centre (NC). The zones are composed of epitheliod and giant cells.

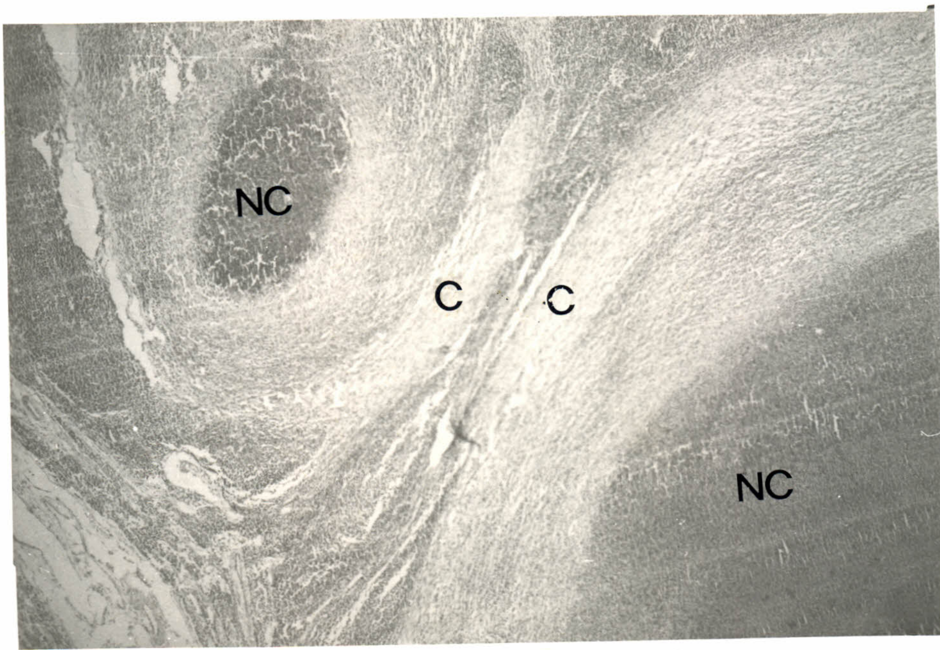


Fig. 21

Multiple capsulated caseous lesions in the draining prescapular lymphnode of a goat 25 days after intradermal infection with 5 x 10^4 cfu of *C. pseudotuberculosis*. NC =necrotic centre, C = capsule. H & E x 100.

Animals sacrificed at days 27 and 30 had one or several capsulated pyogranulomas. In addition, the prescapular node of Goat No. 52 had several circumscribed foci of lymphoid necrosis but without any cellular infiltration.

In Group three animals (infection by scarifaction), abscessed lymphnodes were observed from day nine onwards. However in three of the animals (Nos. 70, 71 and 69) only one lympnode was affected. Lesions of the injection sites were those of scab formation in early stages and none at later stages .

Histologically, there was thickening of capsules due to fibrosis and also conspicuous follicular activity in lympnode sections taken at days 5 and 7. There was also mild PMN infiltration in the medullary sinuses.

Lymphnode sections taken at days 9 and 11 (goat nos. 70 & 68) had multiple granulomas of different sizes distributed randomly in the sections, and extensive fibrosis in the rest of the lymphnode parenchyma. Lesions in the rest of the animals (Nos. 175, 71, 66, 64, 69, and 72) consisted of one large or several typical CLA pyogranulomas, with thick capsules.

Control lymphnodes.

No caseous lesions were seen in all control lymphnodes from all the three groups of goats. Microscopic changes were however seen and varied according to the dose of infection. No changes were observed in animals infected by scarifaction. In animals infected with 5×10^4 cfu, (group 1), changes were observed from days 3-8. There was marked follicular hyperplasia and also oedema of sinuses. Macrophages in medulla had brownish-yellow globular material in their cytoplasm, which resembled haemosiderin, especially in goats Nos.34, 35 and 36. There was also fibrin-like material deposits in the follicles and capillaries

especially in goat No.35. In goat No.36, there was also infiltration of sinuses by PMN. In goat Nos.38-41 there was little or no follicular activity and oedema was not conspicuous. In goat No. 39 there was the globular material in cytoplasm of macrophages in the medulla while in goat No.40, the capsule was thickened and infiltrated with mononuclear cells and PMN There was also a marked accumulation of macrophages with eosinophilic cytoplasm in the medullar sinuses. In animals infected with 1×10^5 cfu, (group 2), contralateral lympnodes had intense follicular hyperplasia in goat Nos.43-48 (days 2-14) and especially in goat Nos.49, 44 and 48 (Fig. 22). Accompanying the hyperplasia was marked oedema. In goat Nos.42-50 there was little or no activity.

Skin Lesions.

The initial lesion observed in the skin injection site was that of diffuse macrophage and polymorphonuclear granulocyte (PMN) infiltration in the dermis, but with multiple foci of a more intense infiltration. The infiltration was also more marked around blood vessels (Fig.23). There was also marked hyperplasia of the stratum spinosum .

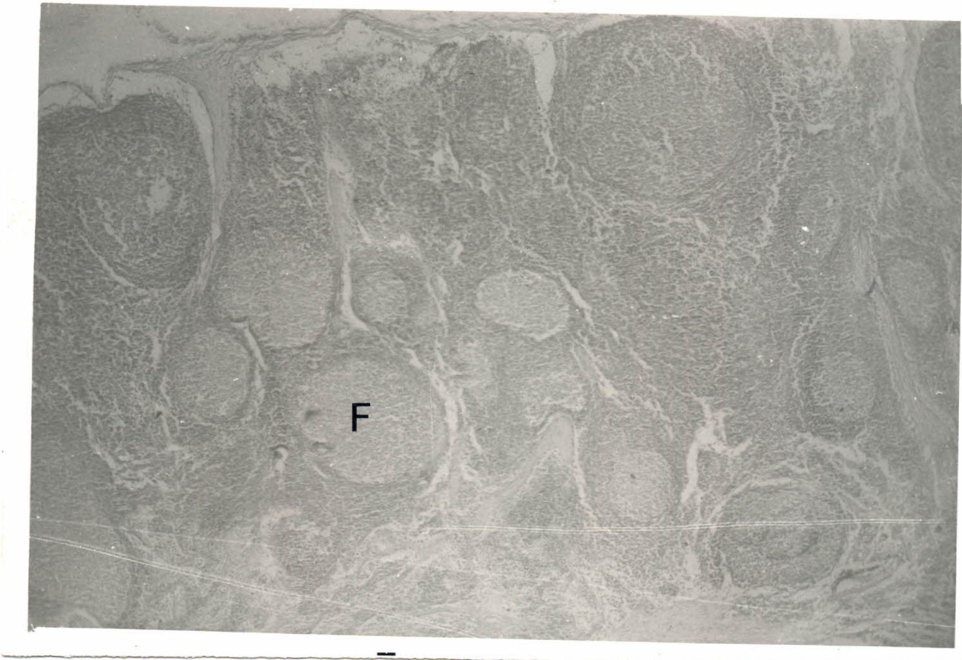


Fig.22

Hyperplasia of lymphoid follicles (f) in a contralateral draining prescapular lymphnode of a goat, 5 days after intradermal infection with 10^5 cfu of *C. pseudotuberculosis*. H & E x 100.

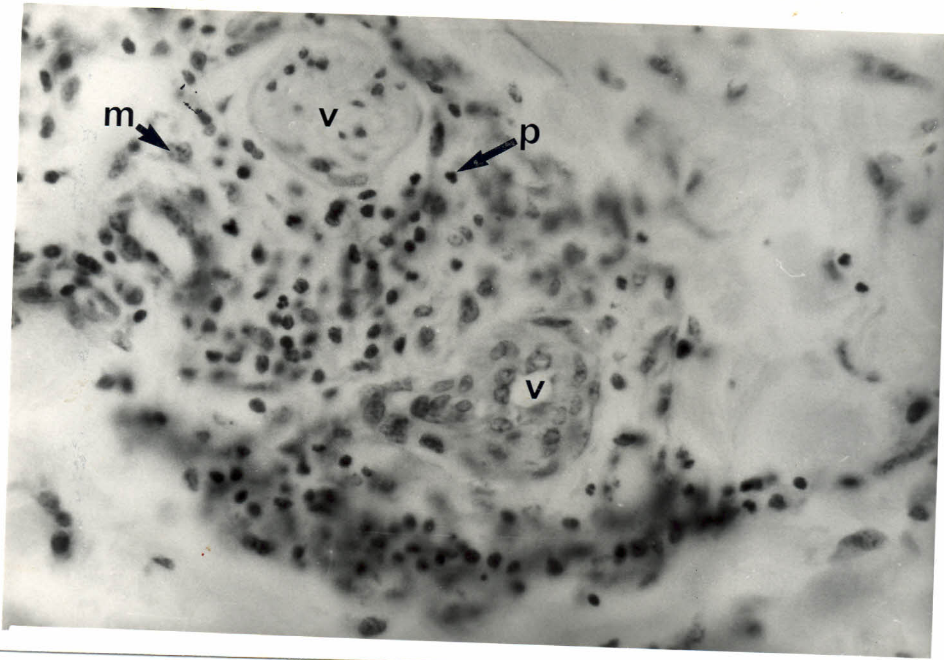


Fig.23

Mononuclear (m) and polymorphonuclear granulocytes (p) infiltration around skin blood vessels (v) in a goat 2, days after intradermal infection with 5×10^4 cfu of *C pseudotuberculosis*. H & E x 400.

Lesions observed later were those of one or more unitised abscesses (Fig 24), composed predominantly of disintegrating mononuclear cells centrally, (Fig. 25). At the periphery, the abscess was composed of a mixture of mononuclear cells and PMN. The abscess was surrounded by a zone of large macrophages, epithelioid and multinucleated giant cells followed by a zone of macrophage, lymphocytes and fibroblasts. Later sections showed capsulated abscesses that had ruptured to the surface. The lesions then healed by formation of granulation tissue (Fig 26).

In goats infected by scarifacation, there was a purulent necrosis of epithelium and mononuclear infiltration in the dermis. The lesions then healed by formation of granulation tissue.

4.4.2. Serological findings.

Positive BAT and HIT titres were recorded on days 17 and 25, 17 and 20, and 15 and 21 in groups 1, 2, and 3 respectively, (Table 9). Goat No. 51 of group 2 (sacrificed on day 20), which had no grossly observable lesions, had a BAT and a HIT titre of 3.0 and 1.2 respectively by day three.

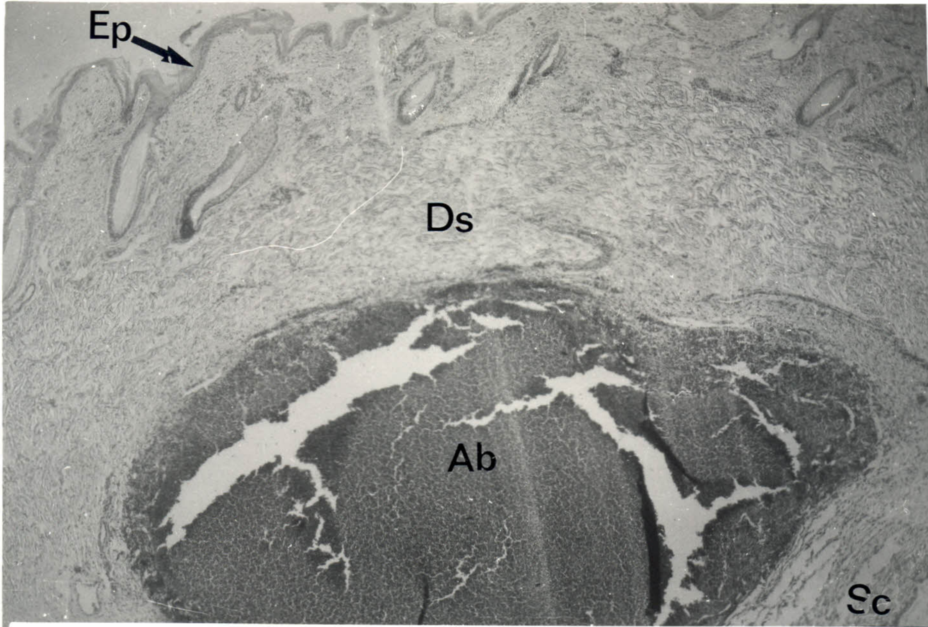


Fig. 24

An intradermal caseous abscess (Ab) in a goat six days after intradermal infection with 5 x 10^4 cfu of *C. pseudotuberculosis*. Ep =epidermis, Ds = dermis and Sc = subcutis. H & E x 40.

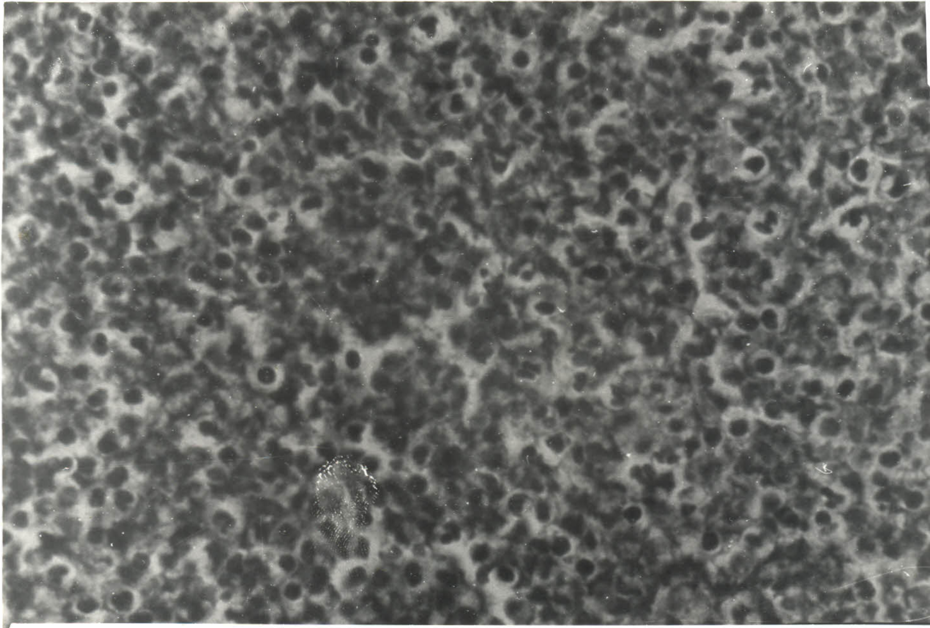


Fig. 25

Central region of a caseous intradermal abscess in a goat showing a predominance of mononuclear cells. H & E x 400.

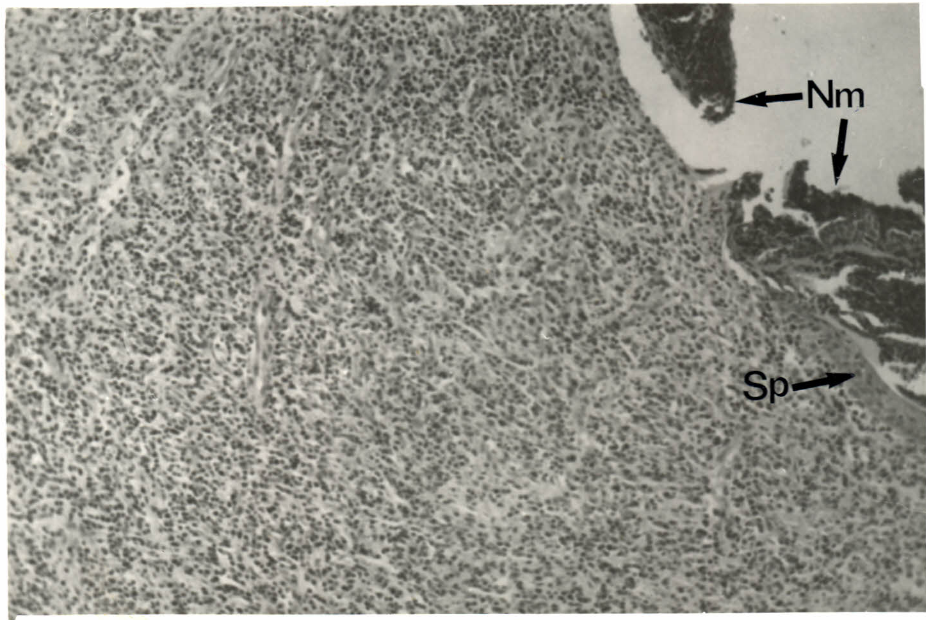


Fig. 26

Healing of a caseous intradermal abscess in a goat by formation of granulation tissue, 34 days after intradermal infection with *C. pseudotuberculosis*. Note the necrotic epithelial material (Nm) and the regenerating stratum spinosum (Sp). H & E x 100.

4.5. Pathogenicity of *C. pseudotuberculosis* fractions and whole cell preparations.

4.5.1. Extraction of the surface lipid.

A sample weight of 10.54 grams of dried cells was extracted with ether. The lipid yield was 0.746 grams. This indicated that lipid content of dried cells was 7.08%. The lipid was orange-yellow in colour and sticky in consistency, at room temperature, and flaky at -20°C.

4.5.2. Pathogenicity of the surface lipid.

Animals injected with the waxy lipid preparation from *C. pseudotuberculosis* showed a marked swelling of draining lymphnodes by the second day post injection. Injection sites were tender and later swollen and hard. By the third week, injection sites were thickened and necrotic. This was followed by ulceration in some sites while others discharged purulent necrotic material. This was followed by scab formation, sloughing off and healing.

Lymphnode enlargement subsided after 2 weeks and sizes were back to normal by the third week. *Actinomyces (Cocynebacterium) pyogenes* was isolated from four discharging sites while others yielded no bacterial isolates.

There was also thickening of control injection sites but without progressing to necrosis and ulceration. No swelling of control draining lymphnodes was observed.

At postmortem, animals sacrificed at days 6 and 9 had thickened injection sites that were black on the cut surface (Due to activated charcoal). Test lymphnodes were enlarged and capsules adhered to the surrounding tissues which had a "cooked" appearance. There were haemorrhages in the lymphnode parenchyma but no abscesses. At day 24 and 30 injection sites were abscessed. In the latter case, the abscess material was waterly and discharged to the exterior through a fistular. The lymphnodes were hard and had a fibrotic

consistency. On day 30, 34 and 42 dark areas were grossly visible on the cut surface of test lymphnodes. The kidneys were enlarged and greyish in colour. In goat No.53, there was a caseous abscess in the left diaphragmatic lung lobe measuring 2cm x 4cm. No bacteria were isolated from the abscess.

Control injection sites were dark on the underside while some control lymphnodes had dark colourations on the cut surfaces.

Histopathology.

Skin sections.

Sections of skin samples obtained at day 6 and 9 post injection showed necrosis of collagen fibres in the dermis and also infiltration by mononuclear cells and PMN. The infiltration had caused considerable thickening of the sections. Blood vessels were congested.

Sections at day 24 had increased cellular infiltration, necrosis of collagen and also acanthosis and hyperplasia of the epidermis. At days 30-40, sections had multiple foci of intense macrophage and PMN infiltration. The rest of skin dermis was diffusely infiltrated with mature macrophage, large macrophages, epitheliod and multinucleated giant cells and fibroblast. Some of the lesions had raptured and discharged to the surface, causing epithelial necrosis.

In test lymphnodes, the initial lesions (day 6-9) consisted of inflammation of a section of the capsule. This section was infiltrated with macrophages, lymphocyte and PMN. Below the inflammed section, there was necrosis of lymphoid tissue which extended towards the medulla (Fig 27). This necrosis was more pronounced along and around trabeculae. Multinucleated giant cells and also macrophages containing eosinophilic globular material in their cytoplasm were present in the necrotic areas. Some macrophages were in the process

of disintegration as evidenced by karyolysis and karyorrhexis of their nuclei. In the rest of the lymphnode section, there was mild follicular activity.

By day 24, follicular hyperplasia was marked, in addition to the necrosis. In later sections (Day 30-40) there was both local extension as well as multiple focal pyogranulomatous reactions in most parts of the sections. The focal reactions were similar to those of CLA lesions and consisted of a necrotic centre of nuclear debri surrounded by a zone of macrophages, epithelioid and numerous giants cells all of which contained droplets of the injected oily material in their cytoplasm (Fig.28). The local extension reactions consisted of infiltratios by macrophages, epitheliod and multinucleated giant cells and few PMN. Lymphnode capsules were thickened and had fused with the cortex. Blood vessels in the capsules showed degenerative changes, of discontinuation of the intima and hyperplasia of the endothelium. There was also vascular thrombosis.

The lung lesion in goat No. 53 consisted of a capsulated granuloma and arteritis of the vessels around the lesion (Fig.29). In the kidney sections, there was focal interstitial lymphocytic infiltration (Fig. 30).

In the control injection sites, sections were thickened due to infiltration by mononuclear cells and also proliferation of fibroblasts. Among the mononuclear cells were multinucleated giants cells. There was generally no reaction of the epithelium.

Control lymphnodes initially had inflammation of the capsule and also circular plaque-like empty areas of different sizes in the cortex and along trabeculae. Later there was marked follicular hyperplasia.

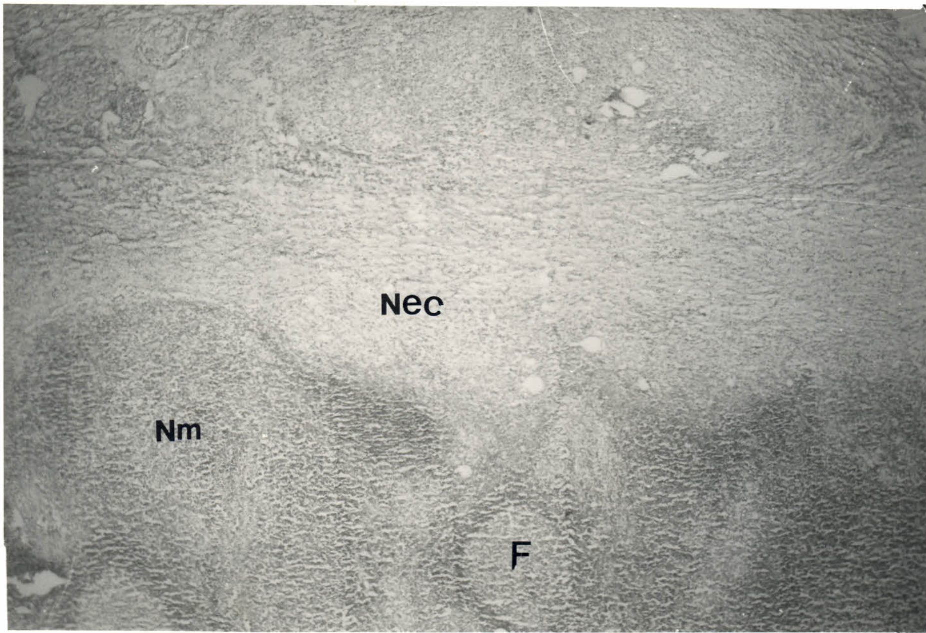


Fig.27

Initial lesion of lymphoid necrosis (Nec) and infiltration in a prescapular lymphnode cortex of a goat 6 days after intradermal injection with the surface lipid of *C. pseudotuberculosis*. Nm = normal tissue, F = lymphoid follicle. H & E x 40.

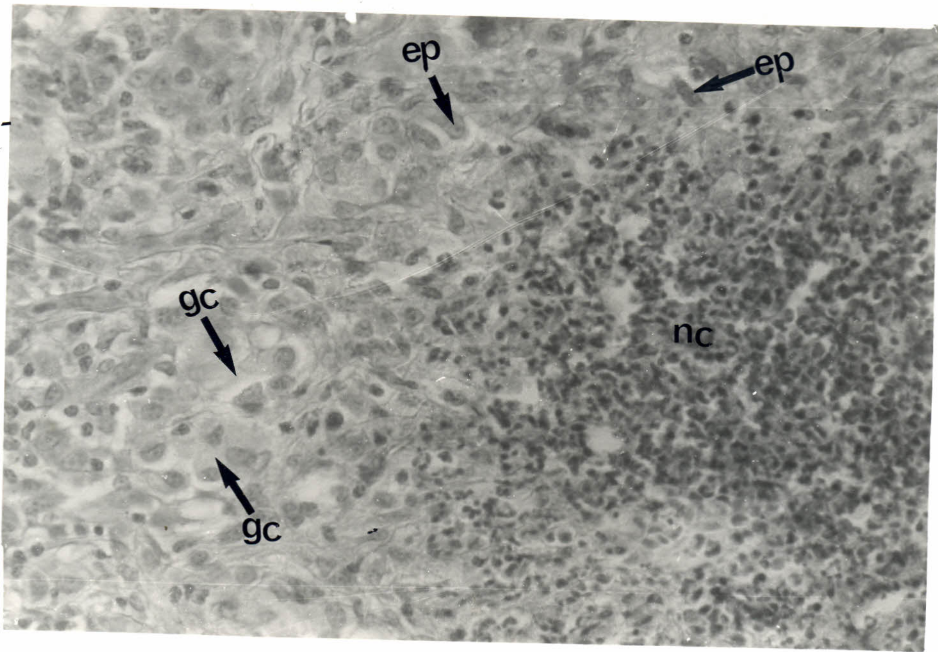


Fig. 28

A pyogranulomatous lesion composed of a necrotic centre (Nc) of macrophages and polymorphonuclear granulocytes, surrounded by a zone of epitheliod (Ep) and giant cells (Gc) in a lymphnode of a goat, 30 days after intradermal infection with the surface lipid of *C. pseudotuberculosis*. Note the droplets of the material in the cytoplasm of the giant cells. H & E x 400.

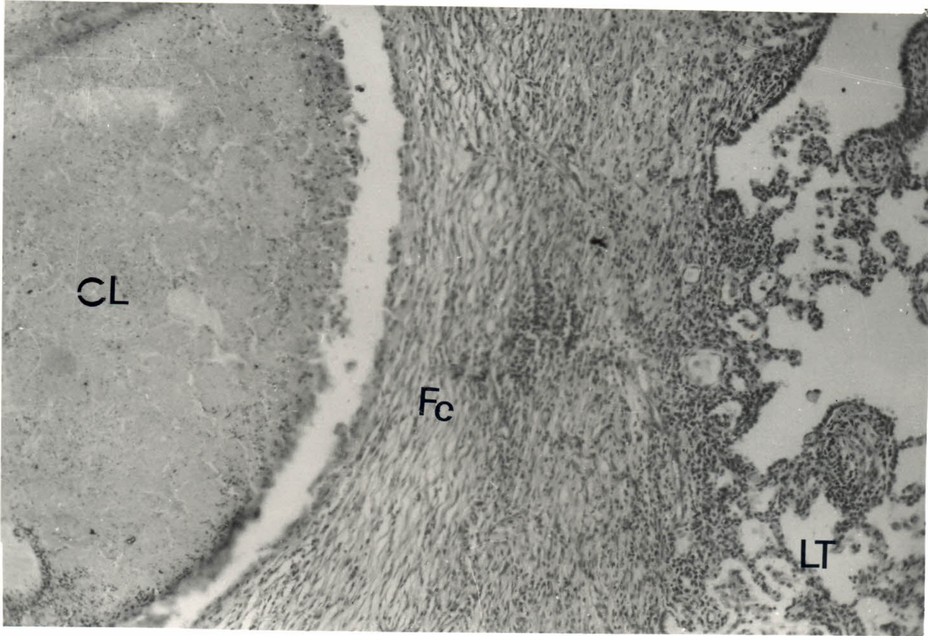


Fig.29

Caseous lesion (CL) in the lung of a goat injected intradermally with the surface lipid of *C. pseudotuberculosis*. FC = fibrous capsule, LT = Lung tissue. H & E x 100.

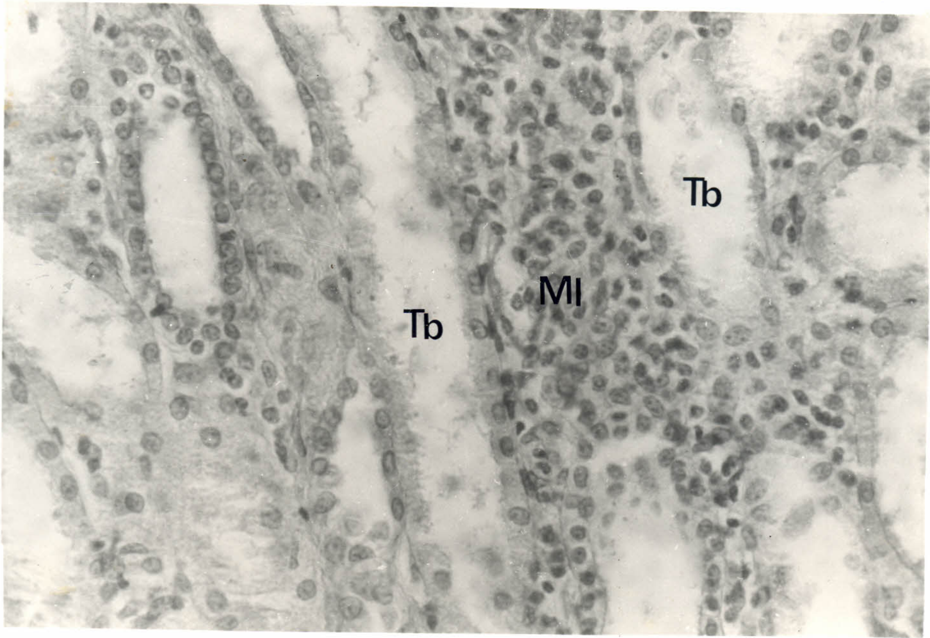


Fig. 30

Focal interstitial mononuclear infiltration (MI) in kidneys of a goat injected intradermally with the surface lipid of *C. pseudotuberculosis*. Tb = tubule. H & E x 400.

4.5.2. Extracellular toxin.

Clinical observation and gross pathology.

There was swelling of test injection sites and draining lymphnodes by the 2nd day. The swelling of injection sites was due to oedema. The swelling increased and was quite marked by day eight and one animal was discharging caseous pus from injection sites. Lymphnode swelling subsided by the 2nd week but injection sites were discharging pus. No bacteria was isolated from the pus. In later stages of observation, (day 17 -35), injection sites were ulcerated and necrotic.

At post mortem, early sacrifices (day 9-24) had haemorrhage, congestion and oedema of test lymphnodes and the surrounding subcutaneous tissues. There was also fibrinous adhesions between test lymphnode capsules and surrounding tissues. At injection sites there was intradermal caseous material from which no bacterial isolates were recovered. Animals sacrificed later, (Day 30 and 35) had ulceration and necrosis of injection sites and in both animals (56 and 57) there were grossly visible abscesses in both test prescapular and precrural lympnodes.

Histopathology.

Sections of samples taken at day 9 had necrosis of collagen fibres and multiple foci of infiltration by macrophages and PMN amidst a diffuse infiltration by large macrophages, epithelioid and giant cells. A section of epithelium was necrotic and had purulent material on the surface. The intact epithelium had marked acanthosis and hyperplasia of stratum spinosum (Fig. 31). There was also furunculosis, haemorrhage and embolism. Samples taken at day 24 had large abscesses at both injection sites and also acanthosis, hyperplasia and necrosis of epithelium. In later samples, there was diffuse granulomatous reaction with

complete destruction of collapse tissue down to the subcutis and also extensive necrosis of epithelium.

In test lymphnodes, there was oedema, inflammation of the capsule and locally extensive lymphoid necrosis. There was also plaque-like empty foci in the necrotic areas and along trabeculae indicating extracellular spaces occupied by the toxin-oil emulsion. In animals sacrificed later (Day 30 and 35) there were multiple foci of pyogranulomatous reaction similar to those observed in animals injected with the surface lipid. The foci composed of either necrotic pink material with nuclear debris or an accumulation of macrophages and PMN surrounded by epitheliod and giant cells (Fig. 32). Some foci had a peripheral fibroblast zone. Other parts of the sections had lymphnoid necrosis and fibrosis. The control skin sections showed increased cellularity with resultant thickening, due to mononuclear cell infiltration, but no necrotic changes. Control lymphnodes initially had no lesions but later had follicular hyperplasia.

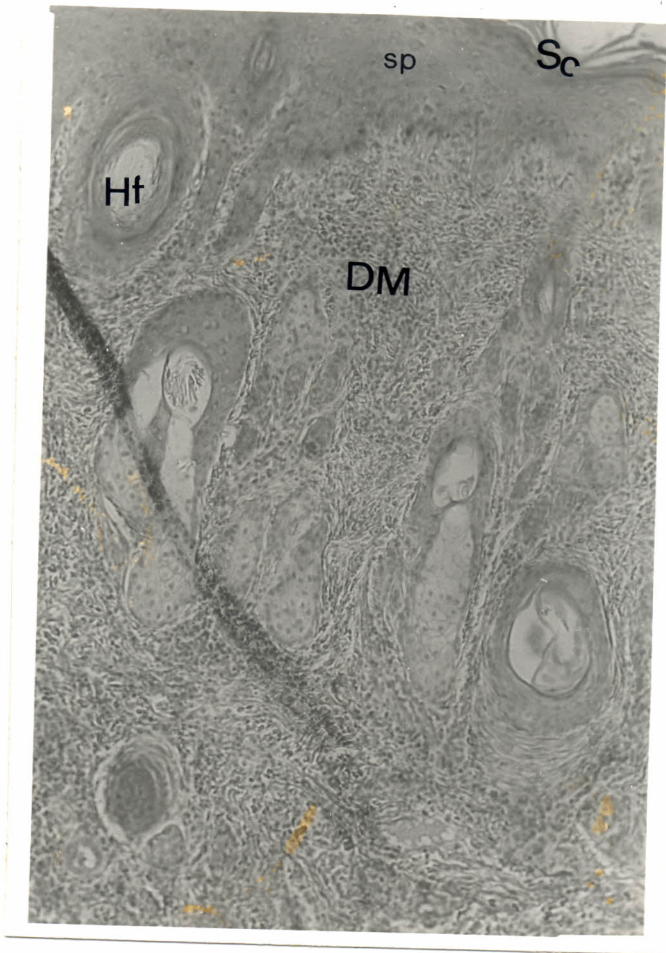


Fig.31

Diffuse infitration with macrophages and polymorphonuclear granulocytes of the skin dermis (Dm) of a goat injected intradermally with the exotoxin of *C. pseudotuberculosis*. Note the hyperplasia of the stratum spinosum (Sp) and the hyperkeratosis of the stratum corneum (Sc). Hf = hair follicle. H & E x 100.

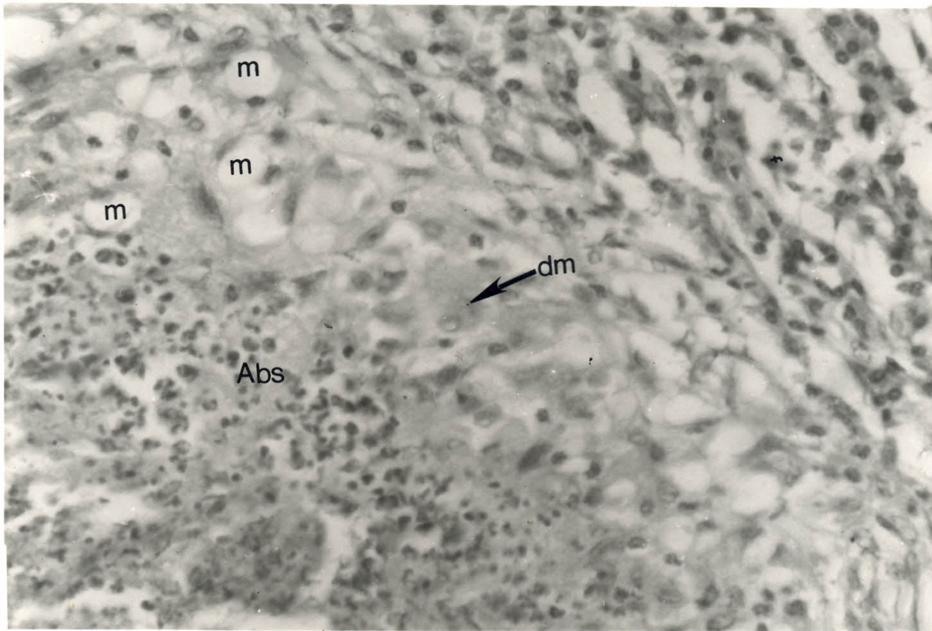


Fig. 32

Lymphnode abscessation (Abs) in a draining lymphnode of a goat injected intradermally with the exotoxin of *C. pseudotuberculosis* emulsified in incomplete Freund's adjuvant. Note the ring of macrophages at the periphery, laden with the injected material in their cytoplasm (m) and some in the process of degeneration (dm). H & E x 630.

4.5.3. Heat inactivated toxin.

Injection sites were slightly swollen by day four. This swelling subsided and was not detectable by day 10. Draining lymphnodes were not palpably enlarged at any stage of the experiment. At postmortem, lymphnodes had black zones on the cut surface and injection sites were black from the underside surface.

Histopathology.

Test and control skin sections were initially thickened due to infiltration by mononuclear cells and giant cells especially around activated charcoal particles. By day 30 and 35, no lesions were detectable. Both test and control lymphnodes showed only mild follicular hyperplasia.

4.5.4. Heat killed, formalin killed, and ether/ethanol extracted *C. pseudotuberculosis* cells

Lymphnodes and injection sites were swollen by day four but lymphnode swelling subsided by day eight. Injection sites were by this time abscessed and some were discharging purulent material. This discharging was followed by scab formation, sloughing off and healing without scar formations. At postmortem, intradermal caseous abscesses were observed in animals sacrificed early in the cause of the experiment. No lymphnode abscesses were observed.

Histopathology.

Skin sections had a single confined intradermal abscess surrounded by macrophages and epithelioid cells, lymphocytes and plasma cells. Some abscesses also had a peripheral

fibroblast capsule. There was no reaction in other parts of the sections.

Lymphnode section showed only oedema and follicular hyperplasia. The pathological findings in injection sites and draining lymphnodes can be summarised in table 10.

4.5.5. Seroloical findings.

A summary of serological findings is given in Table 11. None of the animals injected with the lipid wax extract and with the heated toxin, seroconverted to either BAT or HIT positive. Animals injected with the potent toxin were HIT positive, but BAT negative, from day 24. Animals injected with either heat killed, formalin-killed, or ether/ethanol extracted cells developed positive BAT titres. The titres were however only marginally positive in animals injected with heat-killed cells (titre 2.1).

Animals injected with heat-killed organisms were negative for HIT while those injected with formalin-killed cells had a low titre (0.3). Those infected with ether/ethanol extracted cells had a positive titre (0.6).

Table 10.

Summary of postmortem findings in injection sites and draining lymphnodes of goats injected intradermally with different fractions and preparations of *C. pseudotuberculosis*.

Pathological Findings.

Injection material		Injection sites.		Draining lymphnodes.	
1.	Waxy lipid	1.	Abscessation	1.	Necrosis
		2.	Necrosis	2.	Fibrosis
		3.	Ulceration	3.	Abscessation
2.	Exotoxin	1.	Oedema	1.	Abscessation
		2.	Abscessation	2.	Necrosis
		3.	Necrosis		
		4.	Ulceration		
3.	Heated exotoxin		None		None
4.	Heat- killed cells	1.	Abscessation		None
		2.	Scab formation		
5.	Formalin-killed cells	1.	Abscessation		None
		2.	Scab formation		
6.	Ether/Ethanol washed cells	1.	Abscessation		None
		2.	Scab formation		

Table 11: BAT and HIT titres in goats injected with different fractions and preparations of *C.pseudotuberculosis*.

GOAT NO.	INJECTION MATERIAL	DAY SACRIFICED	BAT		HIT	
			O	T	O	T
78	Lipid wax	6	1.2	0.9	-	-
76		9	0.9	0.9	-	-
77		24	1.8	1.5	-	-
75		30	0.9	0.9	-	-
53		35	1.2	1.2	-	-
55		40	1.2	0.9	-	-
80	Toxin	9	0.9	0.9	-	-
79		24	0.9	0.9	-	0.6
56		30	1.2	1.2	-	0.6
57		35	1.2	1.2	-	0.6
82	Heated Toxin	9	0.9	1.5	-	-
81		24	0.9	0.9	-	-
58		30	1.2	0.9	-	-
59		35	0.9	0.9	-	-
83	Formalin killed cells	24	1.2	3.3	-	0.3
84		30	0.9	3.0	-	0.3
45	Heat-killed Cells	12	1.8	2.1	-	-
60		24	0.9	2.1	-	-
61		36	0.9	1.8	-	-
54	Ether/ethanol extracted cells	18	1.5	3.3	-	-
85		21	0.9	3.0	-	0.6
26		36	0.9	2.7	-	0.6

5.0 DISCUSSION.

5.1. Natural Caseous lymphadenitis.

The commonest site of caseous abscessation in both sheep and goats species was the prescapular lymphnodes. In other parts of the world especially the United States, the lymphnodes of the head region are reportedly the commonest sites of infection (Ashfaq and Campbell, 1979; Burrell, 1981; Batey *et al.*, 1986; Brown and Olander, 1987). The route of infection is thought to be the buccal mucosae, that has been lacerated by rough feed such as hay (Ashfaq and Campbell, 1979). The majority of goats examined in the present work came from arid and semi-arid regions with vegetation comprised mainly of thorny bushes. Superficial scratches inflicted on neck and shoulders during browsing would therefore be the main route of infection, with subsequent high infection rate of the superficial body lymphnodes. Lacerations of the buccal mucosae by thorny bushes while browsing are not likely to be a route of infection unless animals subsequently feed on contaminated feed.

Apart from lymphnodes, lesions were also observed in the subcutaneous tissue and a lesion was observed in the lung of a sheep. However, the disseminated form of the disease, with involvement of visceral lymphnodes and organs was not observed.

The causative agent of CLA, *Corynebacterium pseudotuberculosis* was isolated from the majority of caseous lesions (79.4%), either in pure or in mixed cultures. Other pyogenic and non-pyogenic organism were isolated from 11.1% of the lesions. These other organisms were presumed to be secondary invaders of the lesions, or were contaminants during sampling and isolation. Apart from *C. pseudotuberculosis*, other pyogenic organism such as *Staphylococcus aureus* and *Actinomyces* (*Corynebacterium*) *pyogenes* have experimentally

been shown to cause abscesses in superficial lymphnodes of sheep (Richard *et al.*, 1979). These organisms are however unlikely to cause the caseous granulomatous lesions caused by *C. pseudotuberculosis* (Jubb *et al.*, 1985). A condition of subcutaneous abscessation located close to lymphnodes in sheep (Morel's disease) caused by a Gram positive *Micrococcus* has been reported in Kenya (Shirlaw and Ashford, 1962). No such organism was however isolated from the subcutaneous abscesses in the present investigations.

The pathological manifestation of the lesions was that of unitised capsulated granulomas with necrotic caseous centres. The additional feature of necrosis, in addition to the different cellular compositions qualifies the granuloma into the category of complex granulomas (Adams, 1976). Calcification of lesions is considered rare in goats (Batey *et al.*, 1986) but was frequently observed in the present investigation. The concentric onion-like arrangement of the caseous material was however, not observed in this species. This was in agreement with the observation of Brown and Olander,(1987), that this feature constitutes a major distinction between caprine and ovine CLA. The caseous material, comprising the necrotic centre of the lesions, was found to be composed mainly of dead mononuclear cells as opposed to polymorphonuclear glanulocytes (PMN). This feature is probably responsible for the caseous consistency and the creamy colour of the material. It was also an indication that mononuclear cells (macrophages), are the main host defence cells against the organism. The initial confinement of the spread of the lesions appear to be effected by the epithelioid cells, (derived from macrophages), which are ringed round the necrotic centre. Further confinement of the lesion is effeted by the peripheral fibrous capsule.

Granulomas persist only as long as the inciting agent is present (Spector and

Mariano, 1975; Adams, 1976). Only those microorganisms resistant to phagocytic degradation can produce granulomas (Nelson, 1969; Spector *et al.*, 1970). In CLA, the causative agent would not survive indefinitely in the capsulated lesions. With no viable microorganisms, (9.5% of abscesses were found to be sterile), it is expected that resolution of the lesions would take place. It has been observed that among infected animals, antibody to exotoxin is not necessarily confined to those with lesions (Zaki and Abdel-Hamid, 1971; Nairn *et al.*, 1977; Shigidi, 1978; Brown *et al.*, 1986b). This suggests that some lesions undergo resolution during the early stages of development (Batey, 1986b).

Evidence of resolution of an abscess was observed in the present study. Resolution occurred by formation of granulation tissue. Jolly (1965b), observed that experimentally induced lesions in mice were resolved by specialised macrophages. Lymphnode lesions can also rapture and discharge through the skin (Addo and Eid, 1978) and the lymphnode may then heal by granulation tissue formation.

In the present investigation, caseous lymphadenitis in sheep and goats under nomadic conditions was found to have a prevalence of 1.6% and 7.1% respectively. The prevalence in goats was similar to those reported in other parts of the world but in sheep it was much lower (Ashfaq and Campbell, 1979; Hein and Cargill, 1981; Batey *et al.*, 1986).

5.2. Routes of infection.

The four methods of infection were selected while considering that the natural disease usually involves superficial lymphnodes, from where it can then disseminate to involve deep seated and visceral lymphnodes as well as visceral organs. It is wholly accepted that infection

takes place through skin wounds of varying depths (Nairn and Robertson, 1974; Nairn *et al.*, 1977; Nagy, 1976; Addo and Eid, 1978). The results indicated that the disease in goats can be acquired through introduction of infectious material either subcutaneously, intradermally, or by contact with scarified skin. Infection through intact skin appeared unlikely.

Introduction of infectious material subcutaneously produced the most severe clinical symptoms. The animals were febrile, inappetent and had transient lameness. This severe reaction has been reported by others (Holstad and Teige, 1988b; Pepin *et al.*, 1988). Abdel-Hamid, (1973) and Gameel and Tatour, (1974), observed deaths in goats and sheep respectively when large doses of the organism were injected subcutaneously while small doses produced fever, local abscessation and swelling of regional lymphnodes.

In the present study, swelling and abscessation of subcutaneous injection sites was not immediately accompanied by swelling of regional lymphnodes. Severe inflammatory reaction appeared rapidly at the site, isolating the focus of infection. This was possibly responsible for the delay of the spread of the organism into the regional lymphnodes. Cameron (1972) observed that subcutaneous injection of *C.pseudotuberculosis* in sheep resulted in extensive subcutaneous abscessation but rarely involving the regional lymphnodes.

Introduction of infectious material intradermally produced the most rapid swelling of the regional lymphnode (2 days). Results elsewhere (see incubation period studies) however indicate that abscessation did not occur concurrently with swelling of the lymphnodes, as the latter was mainly due to oedema. Intradermal infection also produced abscessation at injection sites which however, discharged and healed rapidly leaving no trace of infection. Only one goat(No.5) which died three weeks post-infection had abscesses at the infection

sites while another goat(No.6), in addition to abscessation of the regional lymphnodes, developed a subcutaneous abscess close to the prescapular lymphnode. The abscess presumably originated from an affferent lymphatic vessel.

Animals infected on scarified skin developed a mild swelling of infection sites followed by scab formation and healing. Both regional lymphnodes were abscessed at postmortem in all animals. Nairn and Robertson, (1974), failed to induce lymphnode abscessation through penetrating surgical wounds due to excessive scab formation. This apparent contrast may also be due to the fact that Nairn and Robertson (1974), used broth cultures of the organism for infection as opposed to abscess material in the present study. The smeared abscess material remains attached to the site of infection, favouring retention of moisture and nutrients and thus ensuring continued viability of the microorganism (Abdel-Hamid and Zaki, 1972). Nagy, (1976), also reported limited success in reproducing the disease by smearing fresh wounds with abscess material.

Animals infected by smearing infectious material on intact shorn skin developed no lesions in the skin and the draining lymphnodes, contrary to reports by Shigidi, (1979), and Nairn and Roberson,(1974). This method of transmission would require that the organism crosses the cutaneous barrier. Transmission through intact shorn skin is reportedly enhanced by spraying the area with dipping fluid or a suitable defatting agent. In the present work, the shaved sites were swabbed with surgical spirit, a treatment that would have a defatting effect. In absence of abscessation of the lymphnodes it was concluded that transmission through intact skin as reported by others possibly took place through minute shaving cuts.

Haematological findings in all infected groups indicated that the only significant change was a leucocytosis, characterised by a neutrophilia and a subsequent increase in the percentage neutrophil count. The response was more pronounced in groups infected subcutaneously and intradermaly and less pronounced in the group infected through scarified skin. There was no significant change in the group infected through intact skin. There was no significant change in other parameters (Hb, PCV, total plasma protein concentration). Holstad and Teige, (1988b) in addition to a leucocytosis, also observed a decrease in haemoglobin and haematocrit levels in goats infected subcutaneously. Gameel and Tatour, (1974), infected sheep subcutaneously and observed a decrease in red blood cell count, Hb and PCV values. In chronically infected goats, Desiderio *et al.*, (1979), observed a significantly higher total serum protein values than in normal goats, due to increase in gamma-globulins. Plasma proteins were found to increase and to decrease in sheep infected subcutaneously with a high and a low dose respectively (Gameel and Tatour, 1974). In the present study, the value of the haematocrit (PCV), haemoglobin and total plasma protein concentration dropped and remained low throughout the experiment period in all groups including the control group. There was no statistically significant difference in the weekly means of the values between the groups. It was therefore concluded that a uniform factor was responsible for the observed decrease. The unnatural confinement and the change of feed from shrubs to hay was the probable cause. The initial increase in total plasma protein concentration (in all groups) may have been due to dehydration.

The three successful routes of infection produced abscessation of only the superficial lymphnodes. There was no spread to the other lymphnodes or internal organs as reported by Gameel and Tatour, (1974); Holstad and Teige, (1988b); and Pepin *et al.*, (1988). It is

possible that the animals used in the present work had a degree of resistance to infection that confined the infection to the infection site and the regional lympnode.

Caseous lymphadenitis induced by intradermal injection and by skin scarification appeared more typical of the natural disease as it depicted no acute clinical symptoms like those induced by subcutaneous injection. Although signs of general ill health have been reported in animals during the period immediately following the first observation of caseous lymphadenitis in a herd (Holstad and Teige, 1988b), the disease is generally known to take a chronic suppurative course free of acute symptoms such as fever, inappetence, and lameness. The acute clinical symptoms, in addition to the extensive subcutaneous and muscular abscessation in goats injected subcutaneously indicated that this is an unlikely route of natural infection.

5.3. The dose of infection.

The results in the present study indicated that a dose of as low as 100 cfu of *Corynebacterium pseudotuberculosis* can induce CLA in goats when introduced intradermally. Other reports (Ashfaq and Campbell, 1980; Pepin *et al.*, 1988; Holstad and Teige, 1988b) indicate that doses of 1×10^6 cfu are required for experimental induction of CLA. With decrease in the dose of infection, however, especially below 5×10^2 cfu the number of infected lymphnodes observed decreased. At doses of 50 cfu, no CLA lesions were observed. Maki *et al.*, (1985) observed that only about half of the sheep infected with less than 1×10^4 cfu of *C.pseudotuberculosis* developed CLA lesions. In mice, Cameron and Fuls, (1973), found that doses less than 1×10^5 cfu were rarely fatal to mice infected intravenously.

The dose of an organism required to set up an infection is dependent on factors such as the degree of pathogenicity of the organism, the route of infection and the resistance of the host to infection. *C.pseudotuberculosis* produces an exotoxin which acts as a permeability factor (Jolly, 1965a; Carne and Onon, 1978). The permeability factor enhances the spread of the organism from the site of infection to the draining lymphnode. The organism is also a facultative intracellular parasite (Jolly, 1965c; Hard, 1972; Tashjian and Campbell, 1983) and is able to survive and multiply within the host's macrophages (Batey, 1986b). The spreading factor and the resistance to phagocytic degradation would enable a relatively low dose of the organism to establish CLA lesions.

The sources of natural infection are contaminated fomites such as fencing posts, pen walls and floors, soil, straw and faeces or from direct contamination of animals by raptured abscesses. The numbers of the organism available for infection from these sources is unlikely to be high, with the exception of the direct contamination. The period of viability of the organism in the fomites is also limited (Abdel-Hamid and Zaki, 1972; Augustine and Renshaw, 1986). It is therefore important that low doses of the organism be able to establish an infection.

The onset of palpable enlargement of lymphnodes and the frequency of abscessation varied with the dose of infection. Clinical swelling was detected earlier with higher doses but with lower doses it took as long as four weeks. In some cases no clinical swelling was detected at all, such as in groups infected with doses equal to or less than 5×10^2 cfu where only 3 out of 9 lymphnodes were detected as clinicaly swollen. Such lymphnodes, with the exception of one, were also not abscessed at postmortem. This was an indication that

infection did not necessarily lead to lymphnode abscessation. Such animals would however be detected as positive in a serological investigation. In the present results, two animals (Nos. 32 and 25) which had neither clinical swelling nor postmortem lesions, were positive in either BAT or HIT.

5.4. Incubation Period.

There is no available data on the incubation period in the literature for comparison with the present results. Ashfaq and Campbell, (1980) infected goats subcutaneously, intradermally and submucosally and determined the incubation period to be in the range of 41-147 days, calculated from the day of inoculation to the day of incising the abscesses. This method of calculation could not have been accurate since abscesses take time to ripen suitably for incising or lancing. The calculation would however be accurate in so far as dertermining the period after which an infected animal would start contaminating the environment and thereby transmitting the disease.

The method of sequencial sacrificing of infected animals, used in the present study is an accurate method of determining the onset of the CLA lesions, at a stage when detection by either palpation or serology is not possible. The results indicate that whereas the disease is chronic in duration, it is actually subacute in development. The method also allowed observation of the sequencial development of lesions, starting as an accumulation of macrophages and PMN, to progressive formation of several foci of pyogranulomatous lesions. The foci had no capsulation and the cellular contents were identifiable. The foci then coalease , by extension, to form usually one granuloma which becomes capsulated thereby containing the spread of the infection. Internally, containment of the spread would

be aided by the zone of epithelioid cells. There was no significant difference in incubation periods between groups infected with the different doses (1×10^5 and 5×10^4 cfu) or infection methods. The two infection methods are similar in the sense that the infectious material is introduced intradermally although in different quantities and by different means. However, when increasingly low doses of the organism were used (see dose of infection) the period of onset of lymphnodes swelling varied accordingly i.e 2 days with a dose of 1×10^5 and 5×10^4 cfu, 5 days with 1×10^4 cfu, an average of 14 days with 5×10^3 cfu, 21 days with 1×10^3 cfu and 28 days with 5×10^2 cfu (Table I dose of infection). This is an indication that at low doses, the incubation period for development of caseous lesions was dependent on the dose of infection.

The incubation period of a disease is an important diagnostic and epidemiological tool, which can be used to trace the sources of infection. Caseous lymphadenitis is usually introduced into a disease-free herd by newly introduced animals. In Kenya, goat improvement centres provide facilities for hiring out males to farmers. Such males can either take back or bring in infections. Information on incubation period for CLA would therefore allow efficient tracing of newly introduced cases of infection.

5.5. The Pathogenicity of *C. pseudotuberculosis* fractions and whole- cell preparations.

In the present investigation, the two *C. pseudotuberculosis* fractions (Lipid wax and exotoxin), and the whole- cell preparations (Heat-killed, formalin-killed and ether/ethanol extracted cells), produced abscessation at the injection sites but only the lipid wax and the exotoxin produced lymphnode lesions. Heat-killed and formalin killed cells have been

reported to produce sterile intraperitoneal abscesses (Zaki, 1976). This is in agreement with results in the present study. There are however no reports of abscess formation by the lipid wax or the exotoxin.

The lipid wax and the exotoxin are thought to be the major pathogenic factors of *C.pseudotuberculosis* (Carne, 1940; Carne *et.al*, 1956; Jolly, 1965a; Hard 1969a; 1972; 1975; Zaki, 1976; Carne and Onon, 1978 ; Muckle and Gyles, 1983). The pathogenicity of the lipid wax is thought to be twofold. Firstly, the wax allows the organism to resist phagocytic degradation and so survive and multiply as an intracellular parasite (Hard, 1972). Secondly, the substance is thought to be leucocidal. Carne *et al.*, (1956) observed that leucocytes that phagocytosed the surface lipid adsorbed onto activated charcoal underwent rapid degeneration and death. Hard (1975), demonstrated that a lipid extract dissolved in paraffin oil was cytotoxic when phagocytosed by mouse macrophages. Cytotoxicity was assayed by dye exclusion, glycolytic activity and by ultrastructural morphology. The lipid is dermonecrotoxic when injected intradermally in Guinea pigs (Carne *et al.*, 1956) but has no evidence of general toxicity when injected intraperitoneally.

In the present study, the surface lipid of *C. pseudotuberculosis* was tested for ability to produce lesions in lymphnodes. The material, emulsified in liquid paraffin oil, and with activated charcoal was deposited intradermally. The material would presumably move into the draining lymphnode either free or within phagocytes. The former was evidenced by microscopic observation of spaces previously occupied by lipid-oil droplets in lymphnodes. The activated charcoal, in addition to binding the wax, also acted as a tracer, to show that the material moved into the draining lymphnodes. This was quite evident in both test and

control lymphnodes as the nodes had traces of black deposits.

The surface lipid produced pyogranulomatous lesions in draining lymphnodes that were similar to those produced by the live organism. No such lesions were observed in the control lymphnodes. There are no similar reports of *in vivo* lymphnode pathology produced by the surface lipid. A capsulated lung abscess was also observed in one animal. The same animal also had bilateral focal interstitial mononuclear infiltrations in the kidneys. Although it was not possible to determine whether these lesions originated from the intradermally deposited material, the lesions can be produced by entrapment of leucocytes carrying the material (Batey, 1986).

Pyogranulomatous lesions in draining lymphnodes were also reproduced by the potent extracellular toxin. Severe oedema, vascular thrombosis followed by necrosis, ulceration and sloughing off was observed at the injection sites. The oedema and thrombosis, which was attributed to the permeability action of exotoxin, was also reported by Jolly, (1965a). There are no reports however, of lesions in the draining lymphnodes. The inability of the heat treated toxin to produce lesions was an indication that the toxic factor was heat-labile.

Lymphnode lesions produced by both surface lipid and exotoxin were similar histologically. They consisted of both focal and locally extensive pyogranulomatous reactions. The reactions, consisting of degenerate macrophages and PMN surrounded by epitheliod and multinucleated giant cells were similar to the early CLA lesions, before coalescence and capsulation takes place. The ability of the extracellular toxin to cause lympnode lesions may give a partial explanation of the mechanism of host's phagocytes destruction in the

pathogenesis of CLA. The exotoxin has the ability to damage cell membranes. Damage to the phagolysosome limiting membrane (derived from the phagocyte cell membrane) would cause leakage of the degradative enzymes into the cytoplasm. This would lead to self-destruction of the phagocyte.

The heat-killed, formalin-killed, and ether/ethanol washed organism produced lesions in injection sites but not in the draining lymphnodes. There was also none of the clinical swelling of the draining lymphnodes observed with the lipid wax and potent exotoxin. The lesions at the injection sites by the heat-killed and formalin-killed cells can be explained on the basis of the surface lipid on the cells, and those by the ether/ ethanol washed cells on the bases of exotoxin in the cells' protoplasm (Cameron and Smit, 1970). The failure by any of the three preparations to produce lymphnode lesions was an indication that the cells did not move into the draining lymphnodes. Injection sites for lipid wax and exotoxin preparations manifested a marked inflammatory reaction which was not the case with the three cell preparations. The severe inflammatory reaction must have aided the movement of the materials into the draining lymphnodes. Inflammation has been shown to increase vascular permeability and thereby increase lymph flow, prostanglandin levels and polymorphonuclear cells efferent from local lymphnodes (Johnston *et al.*, 1979). It is possible that the lipid wax content of the killed cells was not enough to induce a substantial inflammatory reaction to aid the movement of the cells into the draining lymphnodes. As such, the abscessation remained localised.

5.6. The immune response.

5.6.1. Natural caseous lymphadenitis.

From a total of 57 serum samples from animals with CLA lesions, 50 were positive in BAT (87.7%), 46 in HIT (80.7%), and 51 in either test (89.5%). The proportion of animals detected by the tests was higher when only the 45 animals from which *C. pseudotuberculosis* was isolated were considered i.e 42 in BAT (93.3%) 40 in HIT (88.9%) and 43 in either test (95.5%). This was expected since animals with viable organisms in the lesions are likely to have higher antibody levels. In the absence of viable organisms in the lesions, antibody titres would decline. *C. pseudotuberculosis* was not isolated from the four animals that were negative in either test. In two of the animals, abscesses were sterile while in the other two, other organism were isolated. However, in neither test were negative titres confined to samples from animals from which the organism was not isolated.

Some animals that had no lesions were seropositive in either or both tests. Anderson and Nairn, (1985) and Brown *et al.*, (1986b) also found the proportion of HIT seropositive sheep and goats to be higher than the proportion of animals with abscesses. The descrepancy is attributed to either a subclinical state of infection, with no grossly visible abscesses, or a post-infection status where abscesses had healed. In the present study, there was evidence that:- (1) lesions can resolve, (2) that some doses of infections do not produce lesions, and (3) that lesions appear at least a week before antibodies can be detected. These three observations can account for the observed discrepancy in the present and in previous studies.

The present results indicated that the BAT and HIT have high sensitivity (87.7% and 80.7% respectively) and high specificity (87.9 and 97.2% respectively) in detection of natural

pseudotuberculosis. Holstad, (1986a) reported a sensitivity and specificity of 93% for each test in goats. The reported results were however without the benefit of known negative and known positive sera on the basis of postmortem and bacteriological findings. In the present results, the postmortem and bacteriological status of each animal was known.

The performance of the HIT in the present results, i.e higher specificity than sensitivity was converse to findings by Brown *et al.*, (1986b) who observed a sensitivity and specificity of 98.07% and 71.5% respectively in sheep while Anderson and Nairn, (1985) observed a 13.85% false positive rate.

5.6.2. Effect of the route of infection.

Serum titre values over a 10 week period showed that all animals, except one, infected by either the subcutaneous, the intradermal or by skin scrifaction routes had positive BAT titres by the second week post infection. Animals infected by application of infections material on intact shaved skin did not develop positive BAT titres. The difference in the route of infection in the first three groups therefore, did not influence the qualitative serological response. The findings also confirm those of Holstad and Teige, (1988b) who infected goats subcutaneously and observed BAT seropositive titres by the second week.

Most of the animals infected by either the subcutaneous, the intradermal or by the skin scarifaction route had positive HIT titres by the third week. This was in contrast to reports by Holstad and Teige, (1988b) who observed positive HIT titres five weeks after subcutaneous infection. In the present results the highest titres were observed in animals infected intradermally and in one of those infected subcutaneously. This was an indication that the route of infection may have an effect on the quantitative antibody response.

The BAT was found to detect antibodies one week earlier than the HIT. This was in agreement with findings of Holstad, (1986a; 1986b) and Hostad and Teige, (1988b) who found the BAT to detect both experimental and naturally infected animals earlier than the HIT.

5.6.3. Effect of dose infection.

The results indicate that antibody response is related to the dose of infection. With decrease in the dose of infection, there was both a slower rise in BAT and HIT titres and also lower maximum titres. In animals infected with doses lower than 5 x 10^3 cfu, only 4 out of 8 animals attained positive BAT titres and one animal was only positive between the third and fourth week. The titres were also lower than those in animals infected with doses of equal to and above 5 x 10^3 cfu, which were also in contrast, positive by the second week.

All animals infected with doses equal to and above 5 x 10^3 cfu were HIT seropositive by the third or fourth week, while those infected with lower doses were positive five weeks post infection. Two of the latter group remained seronegative throughout the experimental period i.e, those infected with doses of 100 and 50 cfu respectively. All animals that remained seronegative in either HIT, BAT or both had received infection doses of equal to or below 1 x 10^2 cfu. This included one animal (no. 31) which had a CLA lesion at PM, but remained BAT negative,an indication that low doses may produce CLA lesions without detectable antibody response. Maki *et al.*, (1985) observed that in lambs infected with 1 x 10^2, 1 x 10^3 or 1 x 10^4 cfu, only a half showed an increase in antitoxin ELISA titres. Low challenge doses of equal to or below 1 x 10^4 cfu produced clinically evident lesions and serological response only in half of the animals, and after one month.

These results therefore indicate that serological response, in terms of the rate and extent is affected by the dose of infection. The higher the dose, the faster and higher the serological response.

5.6.4. Incubation period and serological response.

The appearance of CLA lesions did not coincide with appearance of seropositive BAT or HIT titres. Although caseous lesions appeared by days 8-9 in the three groups of animals, seropositivity was not observed until 15-17 days in BAT and 20-25 in HIT .

It has been observed that some naturally infected animals with lesions have no detectable antibody titres , even when the causative agent can be isolated from the CLA lesions. Brown *et al.*, (1986b) observed a 2% failure rate of the HIT to detect infected sheep and goats. Holstad, (1986b) observed that the BAT and HIT each failed to detect 7% of infected goats. In the present study, 7 out of 57 (12.3%) and 11 out of 57 (19.3%) naturally infected sheep and goats were seronegative in BAT and HIT respectively. Of these, three and five animals respectively had viable *C. pseudotuberculosis* in the lesions. The seronegative reactions can partially be explained by the fact that the BAT and HIT titres are seropositive at about one week and two weeks respectively after development of lesions. Maki *et al.*, (1985) found the HIT and the antitoxin ELISA to detect infected sheep 60 - 70 Days and 30 - 60 days respectively after experimental infection.

The serological results in naturally infected animals and in animals used in the incubation period experiment were in agreement that the HIT is likely to "miss" more infected animals than the BAT. The overall implication of these results is that the BAT and the HIT tests are of little value in detection of CLA lesions, during the period 1-2 weeks

after development of lesions.

One experimental animal in group 2 (Goat No. 51) had positive BAT and HIT titres by one week post infection, although the animal was seronegative on pre-infection testing. Postmortem findings in the animal had revealed only a microscopic lesion in one of the test lymphnodes. The lesion was characterised mostly by fibrosis, an indication of a previous infection, which may have resulted in resolution of the lesion. The animal resisted infection (by a dose of 10^5 cfu), and also had a faster antibody response. This was an indication that animals previously infected by *C. pseudotuberculosis* may resist subsequent challenge. The kinetics of the serological response indicated a typical anamnestic response. Brown *et al.*, (1986a) vaccinated goats with a toxoid vaccine and detected HIT titres one week after intradermal challenge with live *C.pseudotuberculosis*.

5.6.5. Serological response to fractions and whole- cell preparations of *C. pseudotuberculosis*.

Antibodies were not detectable by either BAT or HIT tests in sera from animals infected with the surface lipid of *C. pseudotuberculosis*. This was in agreement with report by Maki *et al.*, (1985) who found that the lipid had no antigenic activity in ELISA immunoassays. Extraction of the lipid with ether/ethanol did not alter the immunogenicity of bacterial cells in mice (Cameron and Fuls, 1973). Thus, although the lipid is toxic to host's leucocytes, it does not seem to provoke any antibody response.

The lipid does not therefore play a role in humoral resistance against the organism. Heat inactivated toxin elicited no antitoxin response but the potent toxin did. Similarly, the phospholipase enzymatic activity of exotoxin has been found to be heat-labile (Goel and

Singh, 1972; Kuria, 1984). The results of the present work indicate that in addition to loss of enzymatic activity, heat also destroys the antigenicity of *C. pseudotuberculosis* exotoxin. No HIT titres were detected in animals infected with heat-killed cells.

Antibodies to heat-killed, formalin-killed and ether/ ethanol extracted cells were detected by BAT. Titres to ether/ethanol extracted cells were significantly higher whereas those to heat-treated cells were only marginally positive. Holstad *et al.*, (1989) detected no BAT antibodies in goats immunised subcutaneously with formalin-killed cells. However, subsequent challenge showed that the vaccinated animals had BAT titres detectable one week earlier than non-vaccinated ones, an indication that the killed vaccine had elicited some B-cell activity. The high BAT titres observed in animals infected with ether/ethanol extracted cells confirm observations by Cameron and Fuls, (1973) that extraction of cells with ether/ethanol does not alter the immunogenicity of *C. pseudotuberculosis* cells. HIT titres were also detectable in animals infected with formalin-killed and also ether/ethanol extracted cells. This response was attributed to intracellularly located exotoxin (Cameron and Smit, 1970) whose antigenicity was not affected by either treatment. Titres in animals infected with ether/ethanol extracted cells were however higher. Carne, (1956) reported that light extraction of *C. pseudotuberculosis* cells with petrol/ether did not alter the viability of the cells. The viability of the cells was not however retained after 18 hrs of extraction with ether/ethanol. This was supported by the fact that abscesses at injection sites were sterile and no CLA lesions were observed. The BAT and HIT titres in animals infected with the extracted cells were higher than those in animals infected with other cell preparations. This was an indication that inactivation of *C. pseudotuberculosis* by ether/ethanol extraction preserved the antigeniciy better than either heat or formalin treatment. It is also possible that the removal of the surface lipid by the extraction process exposed the immunogen (epitome) better to the host's

immune system.

6. Conclusions.

The following are the main conclusions from the present study:-

6.1. Caseous lymphadenitis in Kenya is an important disease in goats (prevalence 7.1%) but not in sheep (prevalence 1.8%).

6.2. Abcesses in CLA lesions are composed of mainly dead mononuclear cells as opposed to dead polymorphonuclear granulocytes. This may explain the caseous, and not fluidy nature of the pus.

6.3. Serological evaluation indicated that the BAT and the HIT tests have high sensitivity and specificity in detecting naturally infected and non-infected animals.

6.4. CLA can be induced in goats through either subcutaneous, intradermal injection or by smearing caseous abscess material on scarified skin. Infection through intact skin was found to be unlikely.

6.5. Infection through the intradermal and the skin scarification routes produced CLA that was clinically more typical of the natural disease.

6.6. The main haematological changes associated with *C. pseudotuberculosis* infection is a leucocytosis characterised by a neutrophilia.

6.7. Antibodies to *C. pseudotuberculosis* are detectable two weeks post-infection.

6.8. The minimum dose for induction of CLA in goats through the intradermal route is around 1×10^2 cfu of *C. pseudotuberculosis*.

6.9. Animals infected with low doses may develop CLA lesions but without production of detectable antibody titres. Such animals may therefore be detected as "not infected" serologically. However, since the causative agent is a facultative intracellular parasite,

a delayed skin hypersensitivity test (DHS), could be more acurate in detecting such animals.

6.10. The incubation period for development of CLA lesions in goats is around 8-9 days. CLA is therefore subacute in development.

6.11. Since antibodies are not detectable for at least two weeks post infection, for a period of one week after development of lesions animals will be detected as serologically negative. A cell mediated immune response test such as the DHS may however be able to detect infections earlier.

6.12. The surface lipid and the exotoxin of *C. pseudotuberculosis* can independently induce caseous lesions in goat lympnodes and may complement one another in formation of CLA lesions.

6.13. The surface lipid is not antigenic and may therefore have no role in humoral resistance against *C. pseudotuberculosis* infection.

6.14. *C. pseudotuberculosis* cells extracted with ether/ethanol produced a better antibody response (to both surface antigens and the exotoxin) than cells inactivated by either heat or formalin.

6.15. Although the presence of specific antibodies cannot be equated to immunity, it is possible that inactivation of the organism by an organic solvent such as ether/ ethanol extraction may be a better approach to preparation of vaccines against CLA than either formalin or heat inactivation.

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