SERODIAGNOSIS OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* INFECTIONS IN SHEEP.

BY

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Declaration.

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

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This thesis has been submitted for examination with my approval as a University supervisor on behalf of Proffesor Kare Fossum of the Norwegian College of Veterinary Medicine, Department of Microbiology and Immunology , who supervised the work.

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SUMMARY.

In a serodiagnostic investigation on Corynebacterium pseudotuberculosis infection in sheep, the phenomenon of synergistic haemolytic activity of metabolites from C. pseudotuberculosis and C. equi was investigated. It was found that sheep erythrocytes were more rapidly haemolysed when first treated with C. pseudotuberculosis metabolite than when first treated with C. equi metabolite. It was concluded that C. pseudotuberculosis metabolite altered the erythrocytes in such a manner as to make them be subsequently lysed by C. equi metabolite. Neither the metabolite of C. pseudotuberculosis nor that of C. equi alone haemolysed erythrocytes. The haemolysis was inhibited by antiserum to either C. equi metabolite or to C. pseudotuberculosis bacterium.

In ox blood agar the activities of the metabolites were identical to those of the respective bacterial cultures, but in addition to partial haemolysis, C. pseudotuberculosis culture had a beta-haemolytic activity. This beta-haemolysis was not inhibited by C. pseudotuberculosis bacterial antiserum.

When fractionated by gel chromatography, the active substance of C. equi metabolite eluted homogeneously but that of C. pseudotuberculosis

metabolite eluted in a profile suggestive of an aggregate of different molecules. In agar gel immunodiffusion, there was cross-reaction between C. equi metabolite and an antigen of C. pseudotuberculosis but there was none between the metabolites.

Sheep sera were then tested for antibodies to C. pseudotuberculosis using bacterial agglutination test (BAT) and a modified haemolysis inhibition test (HIT). A C. pseudotuberculosis strain passaged in artificial media was found to give an antigen stable enough for use in BAT. Any sheep serum however, was found to agglutinate this antigen. Treatment of serum with Staphylococcal protein A, 2 mercapto-ethanol or adsorption with C. pseudotuberculosis cells did not prevent this agglutination. Agglutination of C. pseudotuberculosis by sheep serum was therefore concluded to be non-specific. Cross-reaction between C. pseudotuberculosis and C. pyogenes in the agglutination test was not detected.

BAT titres were found to increase generally with age of animals and possibly with infection. There was no relationship between BAT and HIT titres but HIT positive samples also had high BAT titres. In either test, there was a high positive correlation between age and the prevalence of seropositive cases in the sheep flocks.

An enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to C. pseudotuberculosis exotoxin was performed and found slightly more sensitive than the HIT. Such a test would be a simple and rapid test for screening sheep for C. pseudotuberculosis infection.

1. INTRODUCTION.

Corynebacterium pseudotuberculosis causes caseous lymphadenitis, and occasionally pseudotuberculous lesions in internal organs, in sheep and goats (Lovell, 1959; Marsh, 1965; Jubb and Kennedy, 1970; Bruner and Gillespie, 1976; Ayers, 1977; Addo and Eid, 1978). The organism also infects other animals, including man, causing disease conditions other than caseous lymphadenitis (Benham *et al.*, 1962).

Caseous lymphadenitis has a worldwide distribution wherever sheep and goats are raised. It is characterised by abscessation of lymph nodes, especially the peripheral lymph nodes. Of these, the precrural and the prescapular nodes are the most commonly affected (Nagy, 1971; Nairn and Robertson, 1974; Awad *et al.*, 1977a). The condition occasionally becomes generalised with involvement of internal lymph nodes and organs.

Caseous lymphadenitis is usually without clinical signs (Marsh, 1965; Jubb and Kennedy, 1970; Bruner and Gillespie, 1976) but when generalised, non-specific signs of a chronic debilitating condition, with emaciation, poor feed conversion efficiency and poor carcasses are observed. Reproductive failure and sometimes death may also occur. The disease is thus an important economic problem in sheep and goat industry.

The epidemiology and control of such a chronic insidious disease requires a reliable serological test, capable of detecting infections in live animals. Various workers have established a number of tests aimed at this purpose. Two such tests are the bacterial agglutination test (BAT) (Lund *et al.*, 1982a) and the haemolysis inhition test (HIT) (Knight, 1978; Lund *et al.*, 1982a & b). These tests were found suitable for measurement of antibodies to *C. pseudotuberculosis* in goats (Lund *et al.*, 1982a) but no reports of similar work in sheep exist. The original aim of this work was therefore to detect *C. pseudotuberculosis* infection in sheep using the BAT and HIT.

The HIT is based on the ability of metabolites of *C. pseudotuberculosis* and *C. equi* to synergistically haemolyse erythrocytes of a number of animal species among them ovine, caprine, bovine and equine species (Fraser, 1961; 1964; Knight, 1978). This haemolysis can be inhibited by antiserum to *C. pseudotuberculosis*. The sequential role of each metabolite leading to lysis of sheep erythrocytes was therefore to be determined together with the specific activity of each metabolite. Each metabolite was then to be fractionated with a view to obtaining pure samples of the active substance. Possible serological cross-reaction

between the two substances was then to be examined. This part of the work was aimed at improving knowledge on the haemolysis inhibition test principle. This would help improve on the procedure of the test to give more accurate and reproducible results.

The BAT is hampered by the tendency for C. pseudotuberculosis to autoagglutinate. The test also lacks a suitable negative control. After examination of sheep sera from a number of flocks for antibodies to C. pseudotuberculosis using BAT and HIT, the specificity of the agglutination reaction was to be examined. Any possible cross-reaction with C. pyogenes in the agglutination reaction was also to be tested. Titres obtained in BAT and HIT were then to be compared and also examined for relationship to some epidemiological factors in the sheep flocks.

Enzyme-linked immunosorbent assay (ELISA) has been used for detection of antibodies to C. pseudo-tuberculosis using cell wall antigens (Shen et al., 1982). No such test has been attempted using the exotoxin of the organism. An ELISA for detecting sheep antibodies to the exotoxin was therefore to be attempted as a possible substitute for HIT.

2. REVIEW OF LITERATURE.

2.1. Corynebacterium pseudotuberculosis.

2.1.1. Isolation.

Preisz and Guinard isolated the organism in 1891 from a kidney abscess in a sheep and Preisz (1894) named it Bacillus pseudotuberculosis ovis. Nocard (1896) described a similar organism from a horse and the organism was hence known as Preisz-Nocard bacillus. The organism has been known under several other names and the nomenclature has been reviewed by Benham et al. (1962). The American Society of Bacteriologists in 1923 adopted the name C. Ovis, a still commonly used synoname. Carne, (1939) however considered the name a misnomer since the organism was not restricted to sheep, neither was it the only Corynebacterium that affected sheep. In Bergey's mannual (1948) the current name Corynebacterium pseudotuberculosis was therefore adopted.

2.1.2. Description of the organism.

Literature on the characteristics of C. pseudotuberculosis prior to 1962 has been reviewed by Benham et al. (1962). The organism is a non-motile, non-spore forming slender rod that shows pleomorphism. In natural lesions it occurs as bacillary and coccoid forms, singly or as filamentous, but in culture it occurs predominantly as a coccobacillus (Carne, 1939; Nadim and Farid, 1973). In electron microscopy, Hard,

(1969) observed squatly ovoid, or elongated cigar and club-shaped rods, with ovoid or round cross-section, measuring 0.55 by 0.40 µm for short forms and 1.2 by 0.50 µm for the longer forms. In smears, the organism forms clusters, resembling chinese letters when different pleomorphic forms are involved, and a palisade pattern when bacillary forms predominate. (Carne, 1939; Daines and Austin, 1931). Branching, true and false, is observed in smears from lesions and old cultures. (Carne, 1939; Nadim and Farid, 1973).

It is agreed that the organism is Gram positive. The larger forms, especially those from lesions show uneven staining (Carne, 1939; Purchase, 1944; Nadim and Farid, 1973) due to the presence of metachromatic granules (Nadim and Farid, 1973). The metachromatic granules are best observed with Neisser's stain (Purchase, 1944; Carne, 1939). Carne, (1939)and Purchase. (1944) found the organism non-acid fast.

The surface of C. pseudotuberculosis cells has a layer of wax-like lipid substance (Bull and Dickson, 1935; Carne et al., 1956; Jolly, 1966; Hard, 1969) which is extractable with organic solvents (Carne et al., 1956; Jolly, 1966) without impairing the viability of the cells, hence it is external to the cell wall, as confirmed by Hard, (1969).

2.1.3. Cultural characteristics.

C. pseudotuberculosis have cultural characteristics that are associated with the waxy surface lipid layer.

The bacterial cells have a propensity to form clumps (Carne, 1939) and to flocculate spontaneously in suspension (Merchant, 1935; Cameron and McOmie, 1940; Awad, 1960; Keskintepe, 1976a). Hard (1969) found the lipid layer to form an enveloping continuity common to all cells in a clump. Some strains have less tendency to form clumps (Cameron and McOmie, 1940; Awad, 1960; Jolly, 1966). Such strains have lower amounts of the lipid substance (Jolly, 1966; Hard, 1969).

In liquid media, static cultures of C. pseudotuberculosis have the ability to grow as a pellicle (Carne, 1939; Dhanda and Singh, 1955; Nadim and Farid, 1973) due to the surface lipid coat (Jolly, 1966). Strains with lower amounts of the lipid substance have a lesser ability to form pellicles (Jolly, 1966).

On solid media, C. pseudotuberculosis colonies have a waxy consistence (Jolly, 1966). They are not easily emulsifiable (Nadim and Farid, 1973) but colonies of attenuated strains are more hydrophilic (Jolly, 1966) due to quantitatively less surface lipid. Barakat et al. (1970) obtained butyrous and emulsifiable colonies after repeated subculturing of strains.

Formation of a yellow pigment has been observed (Carne, 1939; Marsh, 1947; Dhanda and Singh, 1955) when the organism is cultivated on ox, but not on horse or sheep blood agar (Carne, 1939). Carne interpreted this as an ability to concentrate the high carotin content

in ox serum, not found in horse or sheep serum. Being a surface property the yellow pigment should be associated with the surface lipid layer.

2.1.4. Biochemical properties.

These properties have been reviewed by Benham et al. (1962). Acid, but no gas, is produced from carbohydrate fermentation. It is generally agreed that acid is produced from glucose, galactose, and mannose but activity on other sugars vary. Carne. (1939) examined 133 sheep and one cow strains and found all to produce acid from glucose, fructose, galactose and glycerol. The strains varied in their activity on other sugars. Nadim and Farid. (1973) obtained similar results with 60 isolates from sheep. Gelatin is not liquefied (Carne, 1939) although Lovell (1959) claimed slight liquefaction. No definite hydrogen sulphide production has been recorded. Reduction of nitrates to nitrites appear to vary with the host species of origin of strains. Biberstein et al. (1971) found equine and bovine strains able to reduce nitrates but those from sheep and goats were not. On this basis, two biotypes, one nitrate - reducing from equine and bovine, and the other non-nitrate-reducing from sheep and goats were suggested. There is no agreement on urea hydrolysis. Nitarajan and Nilakantan, (1974b) found isolates from sheep, goats and cattle not to

hydrolyse urea but Keskintepe (1976a) observed hydrolysis by majority of isolates from sheep and goats. Change from urease positive to urease negative through attenuation has been reported (Barakat et al., 1970; Burrell, 1979).

2.1.5. Haemolysin and exotoxin production.

C. pseudotuberculosis is haemolytic in blood agar and also produces an exotoxin detectable in the culture supernatant fluid (Carne et al., 1956). In the literature, there is ample evidence that the haemolysin and exotoxin are different substances produced by the organism (Carne, 1939; Lovell, 1959).

Most strains of C. pseudotuberculosis produce a zone of beta haemolysis when cultivated on sheep, ox, horse or rabbit blood agar (Benham et al., 1962). However the exotoxin in culture supernatant fluid does not cause complete haemolysis (Zaki, 1965; Lovell and Zaki, 1966). Lovell (1959) and Carne (1939) had recognised that C. pseudotuberculosis produces more than one haemolytic pattern on blood agar. Afan (1969) observed colonies grown on ox or sheep blood agar to produce first, a narrow zone of beta haemolysis, followed by another zone of alpha haemolysis. Barakat et al. (1970) observed that C. pseudotuberculosis strains lost their beta haemolytic activity and acquired an alpha haemolytic character after repeated subculturing.

Carne (1939), and Burrell (1979), found anaerobic conditions to enhance haemolytic activity of C. pseudotuberculosis but the same conditions (Carne, 1939) did not influence activity of exotoxin, which remained haemolytically inactive. Carne (1939) found haemolysin production to be enhanced by presence of serum in the medium and Soucek and Souckova (1974) found that a substance distinct from exotoxin was haemolytic on blood agar in presence of calf or sheep serum. They referred to the substance as a "serum factor".

C. pseudotuberculosis haemolysin is closely linked to the bacterial cells, and cannot be obtained free in culture filtrate unlike the exotoxin (Carne, 1939). Washed living bacteria injected intravenously in sheep produced haemoglobinuria and icterus followed by death, but injection of exotoxin resulted in acute intoxication and death without haemoglobinuria and icterus. From this, Carne (1939) concluded that haemolysin and exotoxin were different substances. Also, the haemolysin was not inhibited by antibacterial serum and was therefore non-antigenic, but Carne et al. (1956) and Zaki (1965a) found exotoxin to be antigenic.

The nature of C. pseudotuberculosis haemolysin has not been established. Soucek and Souckova (1974) found the "serum factor" substance to decrease the amount of phosphatidyl cholines in calf serum. They suggested the substance to be a phospholipase A.

C. pseudotuberculosis exotoxin is a sphingomyelinase (Soucek et al., 1967; 1971). The enzyme has activity on sphingomyelins of erythrocyte cell membrane, both in vivo (Soucek and Souckova, 1974) and in vitro, (Soucek and Souckova, 1974; Carne and Onon, 1978) and also sphingomyelin of endothelial cell membranes (Carne and Onon, 1978). The action of exotoxin on erythrocytes is not haemolytic (Carne, 1939; Souckova and Soucek, 1972) but in blood agar it has partial haemolysis (Zaki, 1965a). The exotoxin is lethal when injected intravenously in sheep and guinea pigs (Carne et al., 1956). It is also dermonecrotoxic when injected intradermally (Doty et al., 1964; Jolly, 1966).

The exotoxin of C. pseudotuberculosis interacts with metabolites from other organisms to produce varying effects on animal erythrocytes. Fraser (1961) observed that mixed cultures of C. pseudotuberculosis and C. equi on ox, sheep, goat or rabbit blood agar produced wide zones of complete haemolysis. The same effect is produced in ox blood agar by culture filtrates of the two organisms (Knight, 1978). The exotoxin is antihaemolytic to staphylococcal beta-lysin (Fraser, 1964; Zaki, 1965b; Lovell and Zaki, 1966) but potentiative to staphylococcal delta-lysin (Lovell and Zaki, 1966).

C. pseudotuberculosis exotoxin is a low molecular weight glycoprotein (Onon, 1979). In

chromatographic columns, it has been found to elute non-homogeneously (Soucek et al., 1971; Goel and Singh, 1972; Onon, 1979). Production of the exotoxin in culture is influenced by conditions of incubation, as reviewed by Benham et al. (1962). Doty et al. (1964) found maximum concentration in brain-heart infusion broth after 6 - 7 days at 30°C. Maximum exotoxin concentration was found to correspond with a certain culture pH. Rottgardt (1930) found maximum exotoxin concentration to correspond with maximum pellicle development. Pope and Smith. (1932) found aeration to be essential for production of diphtheria toxin and Carne (1940) observed an optimum aeration area : volume ratio for production of C. pseudotuberculosis exotoxin. Exotoxin is inactivated by heat and by filtration through E.K. Pads (Zaki, 1965a).

2.1.6. Antigenicity of C. pseudotuberculosis.

Cell wall and protoplasmic components of C. pseudotuberculosis are antigenic and elicit specific antibodies. Shigidi (1974) immunised rabbits with whole bacteria and detected antibodies in agar gel precipitation using whole bacterial antigen treated with sodium desoxycholate. The antigenic cell wall component appear to be non-protein and non-lipid in nature. It is not affected by treatment with proteolytic enzymes (Cameron et al., 1969; Cameron and Purdom, 1971; Shen et al., 1982), treatment with trichloroacetic acid (Cameron and Purdom, 1971) or treatment with ethanol : ether (Cameron et al., 1969). The antigen

is as effective as whole bacteria in immunising against C. pseudotuberculosis (Cameron et al., 1969) although the natural configuration of the cell wall is essential for optimum biological activity of the antigen. Different strains of C. pseudotuberculosis are identical in their cell wall antigens (Shigidi, 1974; Awad et al., 1977c) and the antigens do not cross react with C. diphtheria (Shigidi, 1974) or C. pyogenes (Shigidi, 1974; Shen et al., 1982) but have a slight cross-reaction with C. renale (Shigidi, 1974). Cameron and Purdom (1971) found a common immunising antigen between the cell wall and protoplasm of C. pseudotuberculosis.

C. pseudotuberculosis exotoxin elicits production of antiexotoxin which neutralises activity of exotoxin in vivo and in vitro (Carne et al., 1956). Exotoxins from different strains isolated from different host species are antigenically identical, (Doty et al., 1964; Awad et al., 1977b; Lovell and Zaki, 1966). The exotoxin of C. pseudotuberculosis does not cross-react antigenically with that of C. diphtheriae (Lovell and Zaki, 1966) or those of C. haemolyticum (Soucek and Souckova, 1974).

No antigenicity has been demonstrated in the waxy lipid substance of C. pseudotuberculosis. The substance is dermonecrotoxic when injected intradermally (Carne et al., 1956) but antibodies capable of neutralising this activity have not been demonstrated.

The substance appears to be the heat-labile pyogenic factor that produced sterile pyogenic lesions when heat-treated C. pseudotuberculosis inoculated subcutaneously (Zaki, 1976). One mycolic acid, corynomycolic acid, also found in the lipid coat of Mycobacteria has been identified in this lipid substance (Diara and Pudles, 1959).

2.1.7. Pathogenicity of C. pseudotuberculosis.

The pathogenicity of C. pseudotuberculosis is associated with the surface lipid substance, the haemolysin and exotoxin production. It has been demonstrated that attenuated strains contain less of the lipid substance than pathogenic strains (Jolly, 1966; Hard, 1969). The substance appears to have both aggressive and protective roles in the pathogenicity of C. pseudotuberculosis. Carne et al. (1956) found macrophages to undergo rapid degeneration, after engulfing the substance. Jolly (1966) postulated that the lipd layer protected the organism by acting as a barrier against post-phagocytic degradation by phagocyte lysosomal enzymes and Hard (1972) observed that in the phagocytic vacuole, the lipid layer of viable bacteria remained intact, acting as a barrier against degradative enzymes.

Production of haemolysin by C. pseudotuberculosis is associated with virulence. Carre and Bigoteau (1908) inoculated virulent strains of the organism

in sheep and observed intravascular haemolysis, haemoglobinuria and icterus. Carne (1939) observed that the extent of this haemolysis depended on the virulence, route of injection, and the number of organisms injected. No haemolysis was observed with heat-treated organisms or the exotoxin. The haemolytic activity of the organism has been found to diminish or disappear through attentuation (Hard, 1969; Barakat et al., 1970).

Pathogenic strains of C. pseudotuberculosis produce exotoxin and this ability is lost through attenuation (Jolly, 1966; Hard, 1969; Burrell, 1979). Pathogenicity of and exotoxin production by attenuated strains can be restored by passaging through sheep (Burrell, 1979). Lovell and Zaki (1966) isolated non-toxigenic strains from deer and cow and suggested presence in an unusual host as the cause of attenuation.

The pathogenic action of C. pseudotuberculosis exotoxin is by enzymatic activity on sphingomyelins of erythrocyte and endothelial cells cell membranes (Soucek and Souckova, 1974; Carne and Onon, 1978). Jolly (1965) found exotoxin to increase vascular permeability and suggested that it had the role of a spreading factor, facilitating spread of the organism from the primary site of infection to regional lymph nodes. Jolly (1966) found an

attenuated strain to produce no fatal or progressive disease in mice. Immunisation of sheep with exotoxin limited spread of organism to regional lymph nodes on subsequent challenge. Jolly (1965) and Zaki (1976) found mice immunised with exotoxin to have local non-spreading lesions but non-immunised mice developed spreading lesions after experimental infections.

2.1.8. Diseases caused by C. pseudotuberculosis in animals.

Reports on diseases caused by C. pseudotuberculosis have been reviewed by Benham et al. (1962) and are summarised as follows:

Host	Disease condition
1) Sheep	a) Caseous lymphadenitis
	b) Caseous bronchopneumonia
	c) Pseudotuberculosis lesions in other organs.
	d) Severe intoxication with intravascular haemoglobinuria and icterus
	e) Arthritis and bursitis in lambs
	f) Mastitis
	g) Abortion.
2) Cattle	a) Suppurative skin lesions
	b) Mastitis
	c) Lymphadenitis
3) Camels	a) Lymphadenitis
	b) Caseous lymphadenitis

4)	Goats	a)	Caseous lymphadenitis
5)	Deer	a)	Suppurative skin lesions
		b)	Caseous lesions in internal organs.
6)	Horse	a)	Ulcerative lymphangitis
		b)	Infertility and genital infection in the mare
7	Mule	a)	Purulent adenitis
8)	Rhinocerous	a)	Chronic, ulcerative skin sores
9)	Pigs	a)	Lymph node infection
10)	Rodents	a)	Laboratory infection reported
11)	Monkey	a)	Organism isolated from skin ulcers
12)	Man	a)	Lymph node infection
13)	Carnivora		No reports
14)	Avian	a)	Infection in duck reported.

Corynebacterium equi.

2.2.1. Isolation.

The organism was first isolated by Magnusson (1923) as the causative agent of foal pneumonia, associated with pyemia. Jensen (1934) isolated it from soils and thought it to be a saprophytic inhabitant of soils. Wilson (1955) and Bain (1963) isolated it from soils in horse paddocks. Ottosen (1945) obtained isolates from cow dung and Woolcock et al. (1979, 1980) and Barton (1980) isolated the organism from faeces and intestinal tract of a number of animal species, with use of selective media. Barton and Hughes (1980) thought the presence of the organism in the soil was the result of contamination by animal faeces.

2.2.2. Description of the organism.

a) Morphology and staining.

This has been reviewed by Barton and Hughes (1980). The organism is quite pleomorphic, varying in length between coccoid and long, curved forms (Barton and Hughes, 1980). In animal material and in solid media the organism is ovoid or coccoid but predominantly bacillary in broth cultures (Woolcock and Mutiner, 1978). Magnusson (1938) described it as a large bacterium which, in pus appeared coccus-like or ovoid. In superficial colonies, the organism resembled Streptococci but in broth and deep colonies, the cells were irregular rods. Branching has been observed in filamentous forms from liquid cultures (Dafaala et al., 1960). In smears, cells tend to have a clumping or palisade arrangement or resemble chinese letters (Wilson, 1955) or L or V shape (Barton and Hughes, 1980, Magnusson, 1938).

C. equi is Gram positive although in old cultures Gram negative forms can be observed (Barton and Hughes, 1980). Reports on acid-fast staining are conflicting. The organism is occasionally acid fast (Bendixen and Jepsen, 1940) and this may be due to strain variation and variation in staining techniques (Barton and Hughes, 1980). Presence of metachromatic granules have been reported (Magnusson, 1923; Merchant, 1935; Karlson et al., 1940; Brevot et al., 1942),

while other reports indicate failure to observe them (Roberts, 1957; Mcdonald, 1942). The granules are best demonstrated in organisms grown in media containing serum (Barton and Hughes, 1980) or in milk (Bruner and Gillespie, 1973). The organism is non-motile but has scant pili (Yanagawa and Honda, 1976). Spore formation does not occur.

C. equi is encapsulated (Dimock and Edward, 1931; Bruner and Edwards, 1941; Woolcock and Mutiner, 1978) by a polysaccharide capsule (Carter and Hylton, 1974; Woolcock and Mutiner, 1978).

Literature on the cell wall composition has been reviewed by Barton and Hughes (1980). C. equi has cell wall chemotype V, with arabinose and galactose as the major sugars. Among the lipid components are mycolic acids similar to those of other Corynebacteria species (Goodfellow et al., 1976).

b) Cultural characteristics.

The colonial morphology of C. equi is generally described as large, irregularly round, with smooth edge (Magnuson, 1923; Bull, 1924; Dimock and Edwards, 1931; Jensen, 1934; Woodroofe, 1950). The consistency has been observed as wet and viscid or mucoid, succulent or butyrous (Barton and Hughes, 1980). Pigment formation occurs which is described as pink (Magnusson, 1938) (Woodroofe, 1950) reddish-yellow (Magnusson, 1938) yellowish, yellowish-red, salmon-pink rose-pink or

brick-red (Barton and Hughes, 1980). Growth characteristics in liquid media appear variable. Majority of reports such as those of magnusson (1923), Craig and Davis (1940), Brevot et al. (1942), McDonald (1942), Cotchin (1943), Woodroofe (1950), Dafaala et al. (1960), indicate pellicle formation but others such as Dimock and Edwards (1941), Merchant (1935), and Karlson et al. (1940) reported sedimentation and turbidity without pellicle formation. Differences in broth compositions used and strain variation may account for the differences (Barton and Hughes, 1980).

2.2.3. Biochemical characteristics.

These have been adequately reviewed by Barton and Hughes (1980). Carbohydrates are not fermented but addition of glucose has a growth-stimulating effect (Jensen, 1934; Bruner and Edwards,1941). Gelatin is not liquefied and serum is not coagulated but enhances pigment formation. The organism is catalase positive. Production of hydrogen sulphide is not in agreement and indole is not produced. Reduction of nitrate and urease production appear variable. Nitalajan and Nilakantan (1974b) observed variation in urease production and Prescott (1981) found production of urease by most strains examined and nitrate reduction by a majority.

2.2.4. Production of diffusible substances.

It is agreed that C. equi by itself is non-haemolytic. Haemolysis is obtained when C. equi is in a mixed culture with beta toxin producing S. aureus, C. haemolyticum, C. pseudotuberculosis (Fraser, 1964; Nitarajan and Nilakantan, 1974a) and with L. monocytogenes (Fraser, 1964; Nitarajan and Nilakantan, 1974a; Nakawaza and Nemoto, 1980). The substance of C. equi that enhances this haemolysis is diffusible (Frazer, 1964) and is obtainable free in culture (Knight, 1978; Lund et al., 1982 a, b).

2.2.5. Antigenicity.

Magnusson (1923) found all his strains to be agglutinated by the same serum and considered them a homologous serological group. Carter and Hylton (1974) prepared C. equi antisera in rabbit and observed no significant difference between strains in indirect haemagglutination. Precipitin tests revealed a single line. Bruner et al. (1939) found C. equi to be highly type-specific in precipitation reaction and this specificity was unaffected by treatment of cells with acid and heat. A species-specific antigen was demonstrable in cells treated with acid at 100°C. This was further confirmed by Karlson et al. (1940).

Bruner and Edwards (1941) determined that C. equi possessed species-specific, group-specific and type-specific antigens. Treatment of the organism

with acid and heat removed the type and group specific but not the species specific antigens. They concluded that type and group-specific antigens were associated with the bacterial capsule and the species specific antigens with the cell wall.

Woodroofe (1950) found 21 isolates of C. equi to be highly type-specific in agglutination and precipitin reactions. The isolates could be divided into 2 groups that did not cross-react by precipitation reaction but all isolates had a common species-specific antigen by complement-fixation test. Prescott (1981) prepared antisera against 97 C. equi strains and identified 7 different capsular serotypes. There was no relationship between capsular serotype and the host species of strain origin. Bruner et al. (1939) had not been able to classify isolates on epidemiological basis by serological typing.

Antibody production against surface antigen of C. equi appear not to take place readily in infected animals. Magnusson (1938) detected no agglutinins in spontaneously infected animals but were detectable in horses after repeated injections of large doses of the organism intravenously. Cotchin (1943) was unable to raise specific agglutinins in rabbits with live or killed C. equi. C. equi share common cell wall antigens with C. pseudotuberculosis and Mycobacterium smegmatis but not C. renale or C. diptheriae (Cummins, 1962)

2.2.6. Pathogenicity.

The pathogenic mechanism of C. equi is unknown. The organism produces a diffusible substance that is synergistically haemolytic with substances from other bacteria (Fraser, 1961, 1964; Nitalajan and Nilakantan, 1974 a; Nakawaza and Nemoto, 1980). Knight (1978) was unable to demonstrate antibodies against the substance in C. equi-infected foals. The role of the substance in the pathogenicity of C. equi has yet to be determined.

The polysaccharide capsule of C. equi is thought to inhibit phagocytosis and to prevent antibody formation (Smith, 1966, Knight, 1969). Barton and Hughes (1980) considers that the capsular slime layer may enable the organism to stick to cells and initiate infection. A number of bacteria form a "glycocalyx" consisting of a mass of polysaccharide and these fibres enable the bacteria to adhere to surfaces such as cell surfaces (Costerton et al., 1978).

In view of the characteristics of C. equi infection cases, Barton and Hughes (1980) consider the organism a saprophytic, opportunistic pathogen. Cases of C. equi infection are either young foals or young pigs, at a time of immature immune apparatus and waning maternal antibodies. Foals at such a stage are also highly prone to respiratory viral conditions. In adult horses, infections are subclinical and in adult pigs, self limiting. Infections in man have been associated with immunosuppressive drug therapy.

Lesions caused by C. equi are the granulomatous type, with the cellular response being predominantly mononuclear with giant cell formation (Rajagopalan, 1937; Woolcock and Rudduck., 1973; Jang et al., 1975; Cimprich and Rooney, 1977; Mckenzie and Donald, 1979). The organism is therefore an intracellular parasite (Barton & Hughes, 1980). Wilson (1955) elicited delayed hypersensitivity reactions in infected horses with C. equi culture filtrate. Pathogenic mechanisms of C. equi appear to afford it intracellular survival.

3.2.7. Diseases caused by C. equi in animals.

These have been adequately reviewed by Barton and Hughes (1980) and are summarised as follows:-

Host	Disease condition
Foals	1) Purulent pneumonia
	2) Suppurative bronchopneumonia
	3) Arthritis with subcutaneous abscesses
	4) Ulcerative typhilitis
	5) Enteritis and ulcerative enteritis
	6) Colitis
	7) Nephritis
	8) Intestinal abscesses
Adult horses	1) Infertility
	2) Genital infection
	3) Abortion
	4) Mastitis
	5) Pulmonary and subcutaneous abscesses

Adult horses(Cont)	6) Arthritis
	7) Lymphadenitis
Pigs	1) Tuberculous lymph node infection, mostly the cervical and sub-maxillary lymph nodes
	2) Pneumonia.
Cattle	1) Pyometra
	2) Chronic pneumonia
	3) Lymph node infection with abscesses
	4) Mastitis, vaginitis, metritis
	5) Ulcerative lymphangitis
	6) Lymphadenitis
Goat	1) Pneumonia
	2) Arthritis
	3) Liver and spleen abscesses
Sheep	1) Chronic pneumonia
	2) Abortion
	3) Pleurisy
	4) Central nervous system lesions
	5) Suppurative bronchopneumonia
Cat	1) Pyothorax and pulmonary abscesses
	2) Granulamatous - lymphadenitis
	3) Abscesses
Buffalo	1) Purulent vaginal discharge
Reptile	Fulminating bacteraemia in American crocodile and alligator.

Man

1) Pulmonary abscesses
2) Necrotising pneumonia
3) Bacteraemia with pulmonary abscesses.

2.3. Pseudotuberculosis in sheep.

2.3.1. Epidemiology.

The disease is considered a problem of old sheep (Marsh, 1965) but lesions have been found in 4 months old lambs (Benham et al., 1962). Both sexes seem to be equally affected although Nadim et al. (1966) reported a higher incidence in old females than old males. No definite breed predisposition has been demonstrated. However, Nagy (1971) found an apparently higher incidence in the merino than in other breeds.

The commonest portal of entry in natural infection is through skin wounds especially those inflicted during shearing (Nairn and Robertson, 1974; Nagy, 1976). Typical lesions of caseous lymphadenitis have been reproduced in sheep (Gameel and Tartour, 1974) and in goats (Ashfaq and Campbell, 1980) through subcutaneous inoculation. The disease however also occurs even in areas where shearing and docking procedures are not carried out (Addo and Eid, 1978). Docking and castration wounds are considered unimportant (Woodruff and Oxer, 1929).

Infection through natural orifices has been suggested. Nagy (1976) achieved infection through oral and vaginal mucous membranes. Mostafa et al.(1973)

recovered the organism from semen of rams with epididymitis and suggested possible venereal transmission. Nairn and Robertson (1974) found that infection can occur through intact skin weakened by shearing.

The source of infection is mainly discharges from raptured affected lymphnodes which can then directly contaminate wool or skin wounds on other animals or contaminate instruments. Nagy (1976) considered contaminated needles the cause of most abscesses. Nairn and Robertson (1974) found contaminated dips capable of acting as sourses of infection. Soil in sheep premises and sheep assembly points can act as reservoirs for infection. Bull and Dickson (1935) recovered the organism from soil in sheep assembly points in a caseous lymphadenitis-endemic areas and Abdel-Hamid and Zaki (1972) demonstrated survival of the organism in soil, straw and water. Daines and Austin (1932) recovered the organism from faeces of healthy sheep.

Transmission by blood-sucking arthropods has been suggested. Nagy (1971) recovered the organism from tick mouthparts but found no correlation between tick infestation and occurence of infections.

Between flocks, the disease may be spread by introduction of new animals from one flock to another. In goat herds, initiation of the disease has often been traced to introduction of a new animal (Ashfaq and Campbell, 1979; Holstad, 1983).

2.3.2. Clinical signs.

C. pseudotuberculosis infections are usually without clinical signs (Jubb and Kennedy, 1970; Marsh, 1965) and apparently healthy animals may be found affected at slaughter (Bruner and Gillespie, 1973). Addo and Eid (1978), Lovell (1959), Nadim and Farid (1973), and Awad et al. (1977a) however found that affected lymphnodes may be palpably enlarged and caseous material or scars may also be detected when affected superficial lymphnodes rupture. In cases of generalised disease, with involvement of internal lymphnodes and organs, signs of chronic progressive illness with loss of condition have been observed. Death, preceded by anaemia and emaciation may occur (Lovell, 1959). Renshaw et al. (1979) encountered a syndrome of thinning in mature ewes resulting from visceral caseous lymphadenitis. Mostafa et al. (1973) observed reproductive failure in ewes associated with infection of lymphnodes draining the genitalia. A case of infertility in a ram was associated with testicular infection (Turner, 1980) and cases of abortion have been reported (Benham et al., 1962).

In the blood picture, Mottelib et al. (1979) observed leucocytosis, monocytosis, lymphopenia, decreased red cell count and lowered blood sugar and

total cholesterol. Zein El-Abdin et al. (1976) observed decreased total protein, albumin, calcium and calcium : phosphorous ratio. In experimental infection, Gameel and Tartour (1974) observed depressed gamma globulins and elevated alpha and beta globulins. In goats, Desiderio et al. (1978) reported elevated total protein but depressed alpha and beta globulins.

At post mortem, the lymphnodes most commonly found affected are the prescapular followed by the precrural (Lovell, 1959; Marsh, 1965; Nagy, 1971, 1976; Nairn and Robertson, 1974; Awad et al., 1977a). When disseminated, internal lymphnodes especially the mediastinal, bronchial and sublumbar are found affected. Metastatic lesions may be found in the liver, lungs, kidneys, spleen and subcutaneous tissue (Marsh, 1965, Jubb and Kennedy, 1970). Lesions have also been found in the brain (Bandopadhyay, et al., 1976) in the heart (Hamir, 1981) scrotum (Williamson and Nairn, 1980) and as lesions of epididymoorchitis (Krishna et al., 1977). The lesion in affected lymphnodes is abscessation of parenchyma with caseous exudate (Marsh, 1965; Jubb and Kennedy 1970; Addo and Eid,1978). The pus is initially putty-like (Marsh, 1965) or butyrous (Bruner and Gillespie, 1976; Lovell, 1959) and later becomes dry and firm (Marsh, 1965) or granular (Lovell, 1959; Bruner and Gillespie, 1976). The pus is non-odorous (Bruner and Gillespie, 1976) and may appear

as foci or may be diffuse in the lymphnode (Lovell, 1959). Calcification may occur (Sharma and Dwivedi, 1976) but is not common (Lovell, 1959). In old lesions the pus is arranged in concentric layers (Bruner and Gillespie, 1976) and this lamination is considered distinctive of this type of lesion. A fibrous capsule sorrounds the lesion (Marsh, 1965; Jubb and Kennedy, 1970; Lovell, 1959). Affected lymphnodes may rupture, leaving a scar as the only evidence of previous infection (Addo and Eid, 1978). Lesions in other organs generally resemble those in the lymphnodes.

Microscopically, the lesions are the granulomatous type, resembling tubercles. A caseous necrotic centre with nuclear debris is sorrounded by a zone of macrophages, lymphocytes and giant cells and this is then encircled by proliferating fibroblasts (Jubb and Kennedy, 1970; Sharma and Dwivedi, 1976). Gram positive diptheroid organisms may be observed in the lesion.

2.3.4. Diagnosis of pseudotuberculosis.

A number of serodiagnostic tests have been established for detection of C. pseudotuberculosis infections in live animals. These tests detect antibodies to the bacterial cell surface, bacterial cell wall antigens, or to exotoxin and include both in vivo and in vitro tests.

a) The agglutination test.

The test detects antibodies to surface antigens of C. pseudotuberculosis but the propensity of the organism to autoagglutinate is a major problem in preparation of suitable agglutination antigen. Cameron and McOmie (1940) prepared agglutination antigen by shaking a slow flocculating strain with beads. Agglutination of this antigen, in tubes, by sheep sera to a titre above 1/20 was considered specific although this was not supported with post-mortem findings. Normal rabbits failed to react but injected rabbits had high titres. Shigidi (1979) using a similarly prepared antigen found that the tube agglutination test failed to detect confirmed cases of natural caseous lymphadenitis, and after 18 months of experimental infection. Awad (1960) stabilised agglutination antigen by use of normal rabbit serum and performed slide agglutination tests. The reactions obtained were declared reliable and practical for diagnosis of caseous lymphadenitis. The tube agglutination test proved unreliable. Shigidi (1974) however failed to obtain a stable antigen with use of normal rabbit or bovine serum but prepared antigen by shaking bacterial cells with ethanol or by subjecting them to a repeated freeze-thawing process. Agglutination reactions were however not sensitive or specific enough. Some non-infected animals reacted and some infected animals failed to react.

Keskintepe (1976b) obtained stable antigens by using a detergent Tween 80. Tube agglutination reactions were specific. Normal sheep and rabbit serum did not agglutinate the antigens but immune rabbit sera did. Use of various concentrations of magnessium chloride failed to produce stable antigens, as they were agglutinated by normal rabbit and sheep serum. Lund *et al.* (1982a) incoporated Tween 80 in the growth medium, cell washing and suspending saline, in the preparation of agglutination antigen. Goats from a naturally infected herd showed a high response to this antigen in a microplate agglutination test. A serum agglutination titre of 1/64 or more was considered positive.

Burrell (1978) found precolostral lamb sera to agglutinate *C. pseudotuberculosis* non-specifically. He concluded the agglutination test was unsuitable for immune surveys in lambs and that precolostral lamb sera should not be used as a negative control in the test.

C. pseudotuberculosis do not cross-react with *C. equi* (Cameron and McOmie, 1940) *C. pyogenes* or *C. diphtheriae* but cross-react slightly with *C. renale* (Shigidi,1974) in agglutination reactions.

b). Haemolysis inhibition test.

Fraser (1961) observed that mixed cultures of *C. pseudotuberculosis* and *C. equi* produced wide zones

of complete haemolysis on blood agar and Knight (1978) used the principle to detect antitoxin to C. pseudotuberculosis in infected horses. C. pseudotuberculosis culture filtrate produced zones of complete haemolysis in blood agar treated with C. equi culture filtrate and this haemolysis was inhibited by serum from C. pseudotuberculosis-infected horses. The accuracy of the test was found to approach 100% in confirmed cases of infection. No inhibition of haemolysis by sera from C. equi-infected foals was observed. Lund et al. (1982a,b) used a microtiter system to detect antitoxin to C. pseudotuberculosis in goats. Sheep erythrocytes treated with C. equi culture filtrate, called sensitizer, were haemolysed on exposure to C. pseudotuberculosis culture filtrate. Sera from infected goats or from kids suckled by infected mothers inhibited this haemolysis. An inhibition titre of 1/16 was considered positive. A high positive correlation was observed in individual animals between this test and the bacterial agglutination test.

c) Enzyme-linked immunosorbent assay.

Enzymes can be conjugated to antibodies or antigens such that the conjugates have both immunological and enzymatic activities. Degradation of a chromogenic or fluorogenic substance by the conjugated enzymes yields an amplication factor which can then be measured for accurate and sensitive detection of the enzyme. This is the basis of enzyme immunoassay

systems first conceived by Miles and Hales (1968). In the immunoassay system, a labelled antibody reacts with a reagent to produce a substance which can then be assayed to measure the unknown. Engvall and Perlmann (1971) used enzymes as labels and quantitated an antigen using an enzyme-labelled antibody and Engvall et al. (1971) used enzyme-labelled antigen to quantitate an antigen using a specific antibody. Engvall and Perlmann (1972) used enzyme immunoassay to quantitate a specific antibody.

Enzyme immunoassays have since then been extensively used for identification or serodiagnosis of diseases caused by bacteria, viruses, parasites and in identification of drugs and metabolites (Bullock and Walls, 1977). There are different types of enzyme immunoassays and these have been reviewed by Voller et al. (1978). Enzyme immunoassays for antigens can be homogeneous (Rubenstein et al.,1972) or heterogeneous Engvall et al., 1971). Heterogenous enzyme immunoassays are used for antigen or antibody and are also referred to as enzymes linked immunosorbent assays (ELISA) (Engvall and Perlmann,1971). Heterogeneous assays differ from homogeneous assays in that they involve at least one step of separation, in which the bound enzyme conjugate is separated from the unbound conjugate to allow measurement of free or bound conjugate (Voller et al., 1978).

ELISA for serodetection of antibodies in disease conditions are indirect and have been based on the standard ELISA of Engvall and Perlmann (1972). Antigen solubilised in a coating buffer, usually sodium carbonate buffer pH. 9.6 (Engvall and Perlmann, 1972) is physically adsorbed onto an insoluble matrix known as a solid phase. The solid phase material most commonly employed is polystyrene, which is considered the most suitable (Bullock and Walls, 1977) and can be in form of tubes (Engvall and Perlmann, 1972; Bruggman et al., 1977) microplates (Voller and Bidwell, 1975) microsticks (Shekarchi et al.,1982) or beads (Konishi and Takahashi, 1982). Test serum in a diluent containing a blocking agent such as Tween 20 (Engvall and Perlmann, 1972) bovine serum albumin (Ruitenberg et al., 1975) or Tween 80 (Ellens and de Leeuhw, 1977) to prevent nonspecific binding,is reacted with the antigen-coated solid phase. Unreacted serum is then washed off, and enzyme-labelled antiglobulin introduced. Any specific antibody fixed by the antigen would then bind enzyme-conjugated antiglobulin. Unbound conjugate is then washed off and the enzyme substrate added. The resulting colour development will be proportional to the amount of conjugate bound, which in turn will be a measure of specific antibody in the test serum. The intensity of colour development is

measured photometrically or visually and values expressed as optical densities, positive or negative, or as end-point titres (Burrells and Dawson, 1982).

Several modifications of this standard ELISA have been used to suit particular requirements or conditions. Camargo et al. (1978) used monospecific antiglobulin - enzyme conjugates to measure antibodies of a specific immunoglobulin class in whole serum but Naot and Remington (1980) found the method inacurate for IgM measurement, possibly due to competitive inhibition among immunoglobulin classes for antigen binding (Townsend et al., 1982). Cross-reaction among the immunoglobulin classes may also occur due to common antigenic determinant sites in the light chains (Engvall and Perlmann, 1972). Townsend et al. (1982) used fractionated sera in measurement of antibodies of specific immunoglobulin classes and subclasses. A monospecific antiglobulin conjugate is then used for each immunoglobulin class or subclass. Duermeyer and Van der Veen (1978), Duermeyer et al. (1980), Naot and Remington (1980) and Naot et al. (1981) used a double antibody sandwitch system to measure IgM in whole serum. In this test system the solid face is coated with antiglobulin to the immunoglobulin class to be measured. This solves the problem of false-negative results due to competitive inhibition for antigen in test sera (Duermeyer et al., 1980). A similar double antibody sandwitch has been used

when the antigen available is impure (Voller et al., 1978) in which case the solid phase is coated with the antigen-specific antibody.

Townsend et al. (1982) used a four layer modification of the standard ELISA to measure antibodies of different immunoglobulin classes and subclasses in whole or fractionated bovine serum. The test system, called antiglobulin ELISA, uses a bridging antibody between test sera and the conjugate. The bridging antibody is antiglobulin to immunoglobulin class or subclass being measured and the conjugate antibody is then antiglobulin to the antiglobulin. Competitive inhibition among IgG subclasses was found to occur when whole serum was used. A similar assay system, but which avoids use of purified antibodies for preparation of conjugate, called amplified ELISA (a-ELISA) was used by Burtler et al. (1980). The conjugate is a complex of enzyme molecules and antibodies to the enzyme. The antibodies to the enzyme are raised in the same species as the test sera so that they will bind to the bridging antibody of the third layer. The system was found highly sensitive in measurements of antibodies of different immunoglobulin classes in whole serum even at very high serum dilutions.

ELISA has been used to detect antibodies to C. pseudotuberculosis in goats and horses (Shen et al., 1982).. using the standard ELISA.

Soluble protein-free C. pseudotuberculosis cell wall antigen was used on the solid phase. The ELISA was found highly specific, sensitive, and slightly superior to the synergistic haemolysis inhibition test (Haemolysis inhibition test). No ELISA has been performed to detect antibodies to C. pseudotuberculosis exotoxin in any species.

d) Agar gel immunodiffusion.

Shigidi (1974) tested sheep for C. pseudotuberculosis infection by agar gel immunodiffusion, using antigen prepared by treatment of whole bacteria with sodium desoxycholate. Most infected sheep were detected by the test but some control sera also gave positive reactions. Awad et al. (1977c) found the test to have a failure rate of 33% for naturally infected sheep and goats and therefore inapplicable in field surveys. Shigidi (1979) again observed some non-specific reactions and attributed them to possible cross-reactions. The antigen was identical in reaction for C. pseudotuberculosis strains from different host species but cross-reacted slightly with C. renale.

Burrell (1980b) used immunodiffusion in agar gel test to detect antibodies to C. pseudotuberculosis exotoxin in sheep and goats. Animals with confirmed infections and kids suckled by infected nannies reacted positively but non-infected ones did not. The test has however, not been put to any sero-epidemiological use.

e) Other serological tests.

Other test in serodiagnosis of C. pseudotuberculosis infection detect antibodies to exotoxin. These tests have not attracted wide use because they are either inaccurate, technically difficult or expensive. The anti-haemolysin inhibition test (Zaki, 1968) detects antibodies to C. pseudotuberculosis exotoxin in sera by the ability of such sera to inhibit the antihaemolytic activity of exotoxin on Staphylococal beta-lysin. Erythrocytes exposed to C. pseudotuberculosis exotoxin are protected from the lytic action of the beta-lysin but in presence of antibodies to exotoxin, this protection is neutralised and erythrocytes are lysed. This test has been found to have a high false negative rate (Nairn and Robertson, 1974; Zaki and Abdel-Hamid 1974; Shigidi, 1979) and also shows prozone effect with high titre equine sera (Zaki, 1968).

The mouse protection test (Zaki and Abdel-Hamid, 1971) is based on the ability of sera from infected animals to protect mice against the lethal action of exotoxin. The test was found to have no false positive reactions and suitable especially for those animals with no external lesions (Zaki and Abdel-Hamid, 1974; Abdel-Hamid, 1975). Also exotoxins from various strains of C. pseudotuberculosis are antigenically related in the test (Lovell and Zaki,

1966). The test is however expensive (Ayers, 1977) and also fails to indicate the degree of exotoxin neutralisation. Mice may die due to inadequate neutralisation and not necessarily due to absence of antibodies, and in mice that survive, sublethal doses of exotoxin may remain.

C. pseudotuberculosis exotoxin is dermonecrotoxic when injected intradermally (Doty et al., 1964; Jolly, 1965) and antibody to exotoxin neutralises this activity. Doty et al. (1964) used this skin neutralization test to detect infected animals and found the test highly sensitive. All suspect animals gave positive reactions although not all had lesions at post mortem. Awad et al. (1977b) however found some control animals to react positively.

Shigidi (1978) used an indirect haemagglutination test to detect antibodies to exotoxin. Formalised erythrocytes sensitized with exotoxin haemagglutinated in presence of antibodies to exotoxin. The test was claimed sensitive and superior to four other tests (Shigidi, 1979) but non-specific reactions occurred at low serum dilution.

The haemolysin inhibition test (Burrell, 1980a) detects neutralisation of haemagglutinating activity of exotoxin by sera containing antibodies to exotoxin. The exotoxin is haemolytic at pH below 6 (Burrell,1979) but the test is performed at higher pH condition. What is observed

as the negative reaction is therefore haemagglutination and not haemolysis. Inhibition of this haemagglutination is taken as a specific inhibition of haemolysis. The test, like the indirect haemagglutination test, shows non-specific reactions at low dilutions of serum, and also prozone effect.

Train (1935) used C. pseudotuberculosis filtrate, termed "preiznocardine", in an attempt to diagnose infections in horses by delayed skin hypersensitivity. Cameron and McOmie (1940) prepared C. pseudotuberculosis culture supernatant concentrate and attempted the test in sheep. The results were found highly variable. Also, immune rabbits gave negative reactions. Carne (1940) tried a test similar to the Shick test in human but found it inefficient. Renshaw et al. (1979) performed a skin test using a reagent from sonicated C. pseudotuberculosis. The reagent was found to be of limited value in detecting animals infected by C. pseudotuberculosis.

3. MATERIALS AND METHODS.

3.1. Bacterial cultures.

Corynebacterium pseudotuberculosis strain NVH* 2586 and Corynebacterium equi strain NVH 3370 were cultured on agar base (Difco, Detroit, Michigan) containing 5% (v/v) ox blood. The blood agar plates were incubated at 37°C under 100% humidity and a 10% carbon dioxide tension for two days. Colonies obtained from these plates were used for inoculating liquid media.

3.2. Preparation of extracellular metabolites.

C. pseudotuberculosis extracellular metabolite was obtained by the method of Doty et al. (1964). Briefly, colonies of C. pseudotuberculosis were seeded into 500 mls of sterile broth of brain-heart infusion (Difco, Detroit, Michigan) in 2 litre conical flasks stoppered with cotton wool. The flasks were incubated without shaking at 30°C for 7 days. A pool of several cultures was then centrifuged at 8,000xg for 10 min. and the supernant fluid was filtered through 0.22 um millipore filters (Millipore corporation, Bedford, Massachusetts). For preservation against microbial growth, sodium azide (Merck, West Germany) was added to a final concentration of 0.01%. This constituted the crude C. pseudotuberculosis exotoxin and was stored at 4°C.

* Veterinary College of Norway culture collection.

C. equi extracellular metabolite was obtained by the method of Lund *et al.* (1982a). *C. equi* was grown in brain-heart infusion broth shake cultures, incubated at 37°C for 5 days. Cell-free culture supernatant fluid which constituted crude *C. equi* metabolite was harvested as described for *C. pseudotuberculosis* and stored at 4°C after addition of sodium azide to a final concentration of 0.01%.

3.3. Preparation of sheep erythrocytes.

One sheep was bled, each time, by jagular venipuncture. The blood was collected into vacutainer tubes containing Ethylene diamine tetraacetic acid (EDTA) and mixed with an equal volume of Alsever's citrate solution. The mixture was allowed to stand overnight at 4°C. Erythrocytes were then harvested and washed three times with phosphate buffered saline (PBS, pH. 7.2) by successive centrifugation for 10 min. at 600xg. Packed erythrocytes were stored at 4°C and used within one week.

3.4. Standardization of crude *C. pseudotuberculosis* exotoxin and *C. equi* metabolite.

Crude *C. pseudotuberculosis* exotoxin and *C. equi* metabolite were titrated against each other to determine the respective lowest concentrations which in combination were required to produce complete haemolysis of 50 ul of 1% sheep Rbcs in 24 hours. To 50 ul of exotoxin serial dilutions in wells of

V-bottomed polystyrene microtiter plates, (Dynatech, London, England) was added 50 ul of undiluted C. equi metabolite and also 50 ul of 1% (v/v) sheep Rbcs. The plates were sealed with tape to prevent evaporation, shaken for 5 seconds on a microshaker and incubated at 37°C for 24 hours. Extent of haemolysis was then read visually using an inverted mirror (Tetertex, Flow Laboratories, Helsinki, Finland). The last well with complete haemolysis was taken to contain one minimum haemolytic dose (MHD) of C. pseudotuberculosis exotoxin.

C. equi metabolite was in turn titrated. Serial dilutions of the metabolite were prepared in wells of V-bottomed microtiter plates and 50 ul of 2 MHD strength C. pseudotuberculosis exotoxin added together with 50 ul of 1% sheep Rbcs. After incubation for 24 hours at 37°C, extent of haemolysis was read and the last well with complete haemolysis taken to contain 1 MHD of C. equi metabolite.

3.5. Determination of activity sequence in erythrocyte lysis.

The activity sequence of C. pseudotuberculosis exotoxin and C. equi metabolite on sheep erythrocytes leading to haemolysis was determined by pre-incubating erythrocytes with one substance before exposure to the other. Erythrocytes of 1% concentration were mixed with 4 MHD of either C. pseudotuberculosis exotoxin or C. equi metabolite and incubated at room temperature. The control preparation consisted of erythrocytes in PBS. Samples of 50 ul were taken from the mixtures at 2 hr

intervals and transfered into wells of V-bottomed polystylene microtiter plates. To samples containing C. pseudotuberculosis exotoxin 50 ul of C. equi metabolite, containing 4 MHD were added, and the same volume and strength of C. pseudotuberculosis exotoxin was added to samples containing C. equi metabolite. Both C. pseudotuberculosis exotoxin and C. equi metabolite each 50 ul and 4 MHD strength were added to the control samples. Plates were then sealed, shaken and incubated at 37°C. The progress of haemolysis was monitored and the time taken for complete haemolysis to take place in each sample recorded to the nearest 15 min.

3.6. Production of specific antisera against C. pseudotuberculosis exotoxin and C. equi metabolite.

Two rabbits were injected subcutaneously with 0.5 ml crude C. equi metabolite emulsified in 0.5 ml complete Freund's adjuvant. Injections were carried out weekly for 5 weeks. The rabbits were then bled by cardiac puncture 2 weeks after the last injection and the sera inactivated at 56°C for 30 minutes and stored at -20°C.

Rabbit antisera against C. pseudotuberculosis exotoxin was raised with washed, live C. pseudotuberculosis strain NVH 2586. A suspension of bacteria was prepared in PBS to give a density of 1.5% packed

cells. Rabbits were then initially injected intramuscularly with 0.5 ml of the suspension emulsified in 0.5 ml incomplete Freund's adjuvant. After 3 weeks the rabbits were given a series of intravenous injections of the suspension without the adjuvant for 9 days divided into 3 groups of 3 days each with two rest periods of 4 days each in between. Increasing doses were used starting with 0.1 ml and ending with 0.9 ml. The rabbits were bled by cardiac puncture 2 weeks after the last injection and sera inactivated and stored at -20°C.

3.7. Test for neutralisation of C. pseudotuberculosis exotoxin and C. equi metabolite by specific antisera.

3.7.1. Neutralisation in Microtiter wells.

Two MHD of either C. pseudotuberculosis exotoxin or C. equi metabolite were incubated with 50 µl of homologous rabbit antiserum serially diluted in PBS in wells of V-bottomed polystylene microtiter plates. Thereafter, 2 MHD of C. pseudotuberculosis exotoxin containing 1% sheep Rbcs and 2 MHD of C. equi metabolite containing 1% sheep erythrocytes, were added to samples containing C. equi metabolite and to samples containing C. pseudotuberculosis exotoxin respectively.

The control preparations comprised of:-

1). Rbcs in a mixture of C. pseudotuberculosis exotoxin and C. equi metabolite.

2) Rbcs in either C. pseudotuberculosis exotoxin or C. equi metabolite

3) Rbcs in PBS.

4) Rbcs in a mixture of C. pseudotuberculosis exotoxin, C. equi metabolite and normal rabbit serum.

Plates were then sealed, shaken and incubated at 37°C for 24 hrs. The samples were thereafter examined for inhibition of haemolysis.

3.7.2. Neutralisation tests in Ox blood agar.

Wells made in blood agar contained in glass troughs were filled with either C. pseudotuberculosis exotoxin, C. equi metabolite or a mixture of both. Rabbit antiserum to either C. pseudotuberculosis, C. equi metabolite or normal rabbit serum was put into adjacent wells approximately 2 mm. away. The troughs were then covered with glass sheets and incubated at 37°C for 48 hrs and regularly examined during the incubation. They were incubated further at 4°C for several days.

3.7.3. Neutralisation tests in blood agar cultures.

Mixed or separate cultures of C. pseudotuberculosis and C. equi were streaked linearly on blood agar plates and incubated for 24 hrs at 37°C in an atmosphere of 10% carbon dioxide and 100% humidity. Thereafter, wells were made in the blood agar approximately 0.5 cm

from the ends of each streak. Wells on one end were filled with rabbit antiserum to either C. pseudotuberculosis or C. equi metabolite while wells on the opposite end were filled with normal rabbit serum. Incubation of the plates was continued for a further 24 hrs and then examined for serum inhibition of haemolysis.

3.8. Test for heat stability of C. equi metabolite.

Crude C. equi metabolite was heated in a boiling water bath for 5 min and then tested for haemolytic action on sheep Rbcs in presence of C. pseudotuberculosis exotoxin as described previously. Unheated C. equi metabolite was included as the positive control.

3.9. Influence of temperature on C. equi metabolite.

Two samples containing 50 ul of 1% sheep Rbcs., 2 MHD of C. pseudotuberculosis exotoxin and 2 MHD C. equi metabolite were put into wells of V.-bottomed microtiter plates and incubated at 37°C. The samples were examined frequently and at the onset of haemolysis one sample was transfered to room temperature conditions (22°C) and incubation continued. The samples were examined after 24 hrs for complete haemolysis of Rbcs.

3.10. Fractionation of metabolites with ammonium sulphate.

Crude C. pseudotuberculosis exotoxin and C. equi metabolite were fractionated by precipitation with ammonium sulphate following the method of Goel and Singh (1972). The metabolites were saturated 100% with ammonium sulphate by adding 320 gms of the salt to

500 mls of fluid. The mixtures were held at 4°C for 12 hrs. Buffy coloured scums formed and were dissolved each in 50 mls of distilled water. Eleven decimal two grammes of ammonium sulphate were added to each of 50 mls of dissolved scum to give approximately 35% saturation. After 12 hrs at 4°C, the precipitates that formed (P35) were separated by centrifugation. The saturation of the supernatants was increased to 65% by addition of 9.6 gms of ammonium sulphate. The precipitates (P 35-65) were collected by centrifugation after 12 hrs at 4°C. All precipitates were redissolved, each in 5 mls of distilled water. The dissolved fractions were dialysed against distilled water to remove ammonium sulphate and then tested for activity on erythrocytes as described previously. The fraction with the highest activity from each metabolite was further fractionated by gel chromatography.

3.11. Gel chromatography.

Samples of C. pseudotuberculosis exotoxin and C. equi metabolite fractions from ammonium sulphate fractionation were applied to a 1.5x100 cm column packed with Sephadex G 100 (Pharmacia Fine Chemicals) and equilibrated with 0.05 M Tris-Hcl buffer, pH 8.0. The packed gel bed height was 90 cm and the column was operated at 4°C. The samples were eluted with the same buffer, at a flow rate of 20 mls/hr. Absorbance, at 280 nm, of the eluate was continuously recorded. Eluted fractions were tested for haemolytic activity and pooled peak

activity fractions of C. equi metabolite were dialysed against distilled water and freeze-dried.

3.12. Test for cross-reactivity between C. pseudotuberculosis exotoxin and C. equi metabolite using specific antisera.

C. pseudotuberculosis exotoxin and C. equi metabolite were tested for precipitation against the homologous and the heterologous antiserum in agar gel immunodiffusion. Both crude and sephadex-purified C. equi metabolite were tested. The test was performed by the microplate method described by Krause and Raunio (1967). The diffusion chamber contained 1% agarose (Litex, Denmark) in PBS, pH 7.2, and samples of 25 ul were applied to the plexiglass matrix basins. Diffusion was allowed to take place in humid chamber boxes at room temperature for 2 days. Thereafter, the plexiglass matrices were lifted off and the slides washed in 0.2 M sodium chloride solution for 48 hrs followed by a further washing in 0.1 M sodium chloride solution for 12 hours to remove unbound proteins. The slides were then washed in distilled water for 12 hrs and the agarose allowed to dry under an airstream. Thereafter the slides were stained with 1% commassie blue for 10 minutes, decolorised with 10% acetic acid in 70% ethanol and then washed in distilled water. After allowing the slides to dry, precipitin bands were recorded.

3.13. Bacterial agglutination test (BAT).

3.13.1. Selection of bacterial organism.

Seven C. pseudotuberculosis strains i.e. NVH 3368, 2586, 3381, 3513, 3534 and 3452, all originally isolated from caseous abscesses in goats, were compared for their agglutination, cultural, biochemical and exotoxin production characteristics. Strain NVH 3368 was a non-haemolytic progeny obtained after subculturing strain NVH 2586 22 times on blood agar. It had the least tendency to autoagglutinate and was used for preparation of antigen for agglutination test.

3.13.2. Preparation of antigen for agglutination test.

This was prepared by the method of Lund et al. (1982a). Colonies from blood agar cultures of C. pseudotuberculosis strain NVH 3368 were inoculated into 50 mls sterile brain-heart infusion broth containing 1% Tween 80 (Difco, Detroit, Michigan) in 100 ml conical flasks stoppered with cotton wool. The flasks were incubated at 37°C, with agitation on a mechanical shaker, for 22-26 hrs. Bacterial cells were thereafter harvested by centrifugation and washed three times with PBS containing 1% Tween 80 (PBS-Tween). Washed bacterial cells were resuspended in PBS-Tween and the concentration adjusted to an optical density of 0.65 ± 0.05 by spectrophotometry at 535 nm wavelength. Merthiolate was added to a final concentration of 0.01% as a preservative

and to inactivate the cells. This suspension constituted the agglutination antigen and was stored at 4°C and used within two weeks.

3.13.3. Collection and preparation of sheep sera.

Serum samples were collected from 10 precolostral lambs, 274 lambs aged 6 months and 764 sheep aged 1 - 9 years. The serum samples were preserved with sodium azide, final concentration 0.01%, and stored at -20°C until required.

3.13.4. Performance of the BAT test.

The test was conducted following the method of Lund et al. (1982a). Serum samples were tested in parallel duplicates with PBS as the control.

Two-fold serial dilutions of test sera in PBS were prepared in wells of U-bottomed polystylene microtiter plates (Dynatech, London, England). Fifty microlitres of antigen were then added to each well. The plates were then sealed with tape and shaken on a microshaker for 5 seconds followed by incubation at 37°C for 18 - 22 hrs. The plates were then unsealed and samples checked for agglutination. The criteria for agglutination was the formation of a coarse rough-edged layer at the well bottom, and that for no agglutination was formation of a smooth button. Serum titers were read as the highest dilution that gave complete agglutination.

3.13.5. Test for specificity of agglutination reaction.

Sera from three adult sheep and one from a precolostral lamb were incubated with Staphylococcal protein A (Sigma, St. Louis, U.S.A), at a concentration of 8 mg of protein A per ml of serum for 1 hr at 37°C. Nine volumes of agglutination antigen were mixed with one volume of serum and also incubated at 37°C for 1 hr. The supernatant sera were then collected and tested for agglutination. Non-treated samples of equivalent dilution were used as controls.

Samples of the same sera were treated with 2-mercaptoethanol and then tested for agglutination. The treatment was performed by the method of Grubb and Swahn (1958). Briefly, equal volumes of 0.2 M 2-mercaptoethanol (Chemische Fabrik, Swizerland) in 0.1 M phosphate buffer, pH 7.4, were added to serum samples diluted 1:2 in the same buffer. Untreated serum samples of equivalent dilutions were prepared as controls. The preparations were incubated in stoppered tubes at room temperature for 48 hrs and then dialysed against the phosphate buffer for 24 hrs. with four changes of the buffer. Control samples were similarly dialysed in a separate vessel. The samples were then tested for agglutination.

3.13.5. Test for cross-reactivity with C. pyogenes.

One sheep was immunised weekly with a 0.5 ml PBS suspension of formalin - killed C. pyogenes (10×10^9

C.F.U) in complete Freund's adjuvant through subcutaneous injections. The sheep was bled two weeks after the fifth injection and the serum tested for agglutination to C. pseudotuberculosis. The preimmunisation serum was used as control. As C. pyogenes cells clump spontaneously, antibody response in the immunised sheep was measured by Enzyme linked immunosorbent assay using a protein-free C. pyogenes cell wall antigen prepared by the method of Shen et al. (1982).

3.14. Haemolysis inhibition test (HIT).

3.14.1. Performance of the test.

The test was performed using a modification of the method of Lund et al. (1982a, b). Duplicate serial dilutions in PBS of 50 ul of test sera were prepared in wells of V-bottomed polystylene microtiter plates. C. pseudotuberculosis exotoxin, 50 ul, containing 2 MHD was added to each well. The mixtures were incubated at room temperature for 2 hrs and thereafter, sheep erythrocytes, freshly suspended to a concentration of 1% in C. equi metabolite containing of 2 MHD per 50 ul, were added in 50 ul amounts to each well. Controls comprised:-

1) Erythrocytes in C. pseudotuberculosis exotoxin.
2) Erythrocytes in C. equi metabolite.
3) Erythrocytes in a mixture of C. pseudotuberculosis exotoxin and C. equi metabolite.
4) Erythrocytes in PBS.

The plates were sealed, shaken and incubated at 37°C for 24 hrs. The samples were then checked for serum inhibition of haemolysis. Serum haemolysis inhibition titres were read as the highest dilution that completely inhibited haemolysis. Titres of 1/4 and above were regarded positive.

3.14.3. Test for reproducibility of HIT results.

Five serum samples with different haemolysis inhibition titres were tested, each 20 times for 5 days. Titre standard deviations, expressed as two-fold dilutions, were then calculated.

3.14.4. Test for specificity of HIT reaction involving C. pseudotuberculosis exotoxin.

One sheep serum sample with a positive HIT titre was tested for reaction of identity to the experimentally produced rabbit anti-C.pseudotuberculosis serum against C. pseudotuberculosis exotoxin in agar gel immunodiffusion. The testing was conducted as described previously by the microplate technique of Krause and Raunio (1967).

3.15. Anti-C. pseudotuberculosis exotoxin ELISA test.

A four layer indirect antibody ELISA system similar to that of Townsend et al. (1982) was used. Ammonium sulphate - precipitated C. pseudotuberculosis exotoxin p^{35-65} in PBS, pH 7.2 was used as antigen. Flat - bottomed polystylene microtiter plates (Nunc, Copenhargen, Denmark) were coated with 100 ul of antigen,

containing 10 MHD per ml, in each well at 4°C overnight. Thereafter, unattached antigen was removed from the plate wells by washing five times with 100 ul. in each well, of 0.85% phosphate buffered saline containing 0.05% Tween 20 (PBS - Tween 20) using a manual washing equipment. The plates were then inverted to dry after which 100 ul of each serum sample diluted 1/250 in PBS-Tween 20 were added into each of two wells. The wells in the first column contained PBS-Tween 20 alone and served as controls.

The plates were then sealed and incubated at 37°C for 2 hrs. Unbound serum was then removed from the wells by washing five times with PBS-Tween 20 as before. Rabbit anti-sheep IgG (Nordic Immunology, Tilburg, Netherlands) diluted 1/1000 in PBS-Tween 20 containing 1% normal goat serum (containing no antibodies to C. pseudotuberculosis exotoxin) was then added 100 ul to each well. Plates were again sealed and incubated at 37°C for 24 hrs.

After another five times washing, 100 ul of goat anti-rabbit IgG, conjugated to Alkaline phosphatase (obtained from B.P. Berdal, Norwegian Defence Microbiological Laboratory. The conjugation had been done by a one-step glutaldehyde procedure) diluted 1/1000 in PBS-Tween 20 containing 1% normal goat serum, were added to each well. Incubation was again done at 37°C for 2 hrs.

The wells were again washed five times and 100 ul of 0.5 mg per ml solution of p-nitrophenyl phosphate (Sigma, St. Louis, U.S.A) in diethanolamine (Merck-Schurchardt, Holsenbrunn,W. Germany) buffer pH. 9.8 (Voller et al., 1980) were added to each well. Development of yellow colour was monitored, and arrested by addition of 50 ul of 4N sodium hydroxide solution to each well, when obvious in wells other than the control wells. The optical density (OD) values of the yellow colour were read at 405 nm using a Multiskan ELISA reader (Titertex, Flow Laboratories, Helsinki, Finland) with blank adjustment made against the control wells.

4. RESULTS.

4.1. Haemolytic activity of C. pseudotuberculosis exotoxin and C. equi metabolite.

In presence of both C. pseudotuberculosis exotoxin and C. equi metabolite, sheep erythrocytes were haemolysed but not when either was absent. The concentration of the active substance in C. equi metabolite appeared to be very low compared to that in C. pseudotuberculosis exotoxin. Crude C. equi metabolite had 16 MHD per 50 ul compared to 32,768 MHD per 50 ul in C. pseudotuberculosis exotoxin. Also when C. pseudotuberculosis was titrated in excess of C. equi metabolite, there were several intermediate stages between complete haemolysis and no haemolysis, which developed to complete haemolysis over time. A similar titration of C. equi metabolite in excess C. pseudotuberculosis exotoxin gave a sharp division between complete haemolysis and no haemolysis.

4.2. The sequence of haemolysis by C. pseudotuberculosis exotoxin and C. equi metabolite.

Figure 1 shows the effect of pre-incubating erythrocytes with either C. pseudotuberculosis exotoxin or C. equi metabolite on haemolysis time. Erythrocytes pre-incubating with C. pseudotuberculosis exotoxin then exposed to C. equi metabolite were more rapidly haemolysed than the control erythrocytes. The time for haemolysis to take place decreased with increase in the

time erythrocytes were incubated with C. pseudotuberculosis exotoxin and reached a minimum of 2 hrs. In contrast, time for haemolysis to take place increased with increased incubation of erythrocytes with C. equi metabolite. This increase reached a maximum before decreasing to a period similar to that of control erythrocytes. It was observed that those erythrocytes incubated with C. equi metabolite retained their bright red colour but those incubated with C. pseudotuberculosis exotoxin darkened and a yellow colour appeared in the supernatant fluid.

4.3. Neutralisation of C. pseudotuberculosis exotoxin and C. equi metabolite activity by specific antisera.

Homologous antisera neutralised activity of C. pseudotuberculosis exotoxin and C. equi metabolite on sheep erythrocytes. Haemolysis inhibition titres for the two rabbit anti-C. equi metabolite antisera were 1/256 and 1/128 respectively. Rabbit anti - C. pseudotuberculosis antisera neutralised haemolysis to a titre of 1/512.

Figure 2 shows activity of C. pseudotuberculosis exotoxin and C. equi metabolite on ox blood agar and the inhibition of this activity by homologous rabbit antiserum. C. pseudotuberculosis exotoxin produced a zone of partial haemolysis. This haemolysis was inhibited by C. pseudotuberculosis antiserum but not

by C. equi metabolite antiserum or normal rabbit serum. The partial haemolysis became more pronounced after refrigeration at 4°C.

C. equi metabolite produced no visible effect on blood agar but a mixture of the metabolite and C. pseudotuberculosis exotoxin produced beta-haemolysis. This haemolysis was completely inhibited by rabbit anti - C. pseudotuberculosis antiserum and partially inhibited by rabbit anti - C. equi metabolite.

Figure 3 shows the effect of rabbit antisera on haemolytic activity of C. pseudotuberculosis culture and of mixed cultures of C. pseudotuberculosis and C. equi. C. pseudotuberculosis produced a narrow zone of beta-haemolysis which was not inhibited by rabbit anti - C. pseudotuberculosis antiserum or by normal rabbit serum. A mixed culture of C. pseudotuberculosis and C. equi produced a wide zone of beta-haemolysis which was inhibited by antiserum to either C. pseudotuberculosis or C. equi metabolite. The extent of this haemolysis measured from the edge of culture, was less on the side of wells with immune sera than on the side of wells with normal sera. At the end of the 48 hours incubation period partial haemolysis covered most of the rest of the plate but was absent around wells containing rabbit anti - C. pseudotuberculosis antiserum.

4.4. Thermal inactivation of C. equi metabolite.

C. equi metabolite was found to be heat labile. Boiling for 5 minutes completely destroyed the activity. The metabolite activity was also found to be temperature dependent since rapid activity took place at 37°C although no activity could be detected at room temperature conditions

4.5. Ammonium sulphate fractions of C. pseudotuberculosis and C. equi metabolite.

C. equi metabolite p^{35} fraction was a greyish-white precipitate partially insoluble in water but soluble in dilute ammonium sulphate solution. Very little activity could be detected in this fraction. C. equi metabolite fraction p^{35-65} was a light brown precipitate soluble in distilled water, to form a dark brown liquid. The activity of this fraction, in MHD, was comparable to that of the crude C. equi metabolite, at the same concentration measured as absorbance at 280 nm.

C. pseudotuberculosis exotoxin fractions p^{35} and p^{35-65} had similar physical characteristics to those of the corresponding C. equi metabolite fractions, fraction p^{35-65} was the most active but less active than the crude C. pseudotuberculosis exotoxin at the same concentrations.

4.6. Sephadex fractionation.

Figures 4 and 5 show elution profiles of C. pseudotuberculosis exotoxin fraction p^{35-65} and C. equi metabolite fraction p^{35-65} from Sephadex G-100 respectively. C. equi metabolite could be separated into two absorbance peaks. Activity was detected under the ascending part of the second peak. The average void volume for the activity peak was 100 mls.

C. pseudotuberculosis exotoxin was separable into two major absorbance peaks. Activity was spread throughout

the absorbance profile but three peaks of activity could be identified. The average void volume for the first fraction with activity was 50 mls.

4.7. *C. pseudotuberculosis* exotoxin and *C. equi* metabolite cross-reaction test.

Figures 6 and 7 show the results of this test in gel immunodiffusion precipitation. *C. pseudotuberculosis* antiserum formed at least four precipitin bands with *C. pseudotuberculosis* exotoxin and also two precipitin bands with crude *C. equi* metabolite (Fig 6). Antiserum to *C. equi* metabolite formed one broad precipitin band with *C. pseudotuberculosis* exotoxin (Fig 7) or with crude *C. equi* metabolite. No precipitin bands could be obtained with Sephadex-purified *C. equi* metabolite against either *C. pseudotuberculosis* antiserum or *C. equi* metabolite antiserum.

4.8. Characteristics of *C. pseudotuberculosis* strains tested for agglutination antigen suitability.

4.8.1. Biochemical characteristics.

Production of acid from glucose and glycerol was common to all seven strains. In addition, strains 3381, 2534 and 3537 produced acid from lactose. Only strain 3381 produced acid from sorbitol, strain 3534 from trehalose and sucrose and strain 3452 from mannitol. All seven strains hydrolysed urea but none reduced nitrates.

4.8.2. Autoagglutination.

Antigens from strains 2586, 3452 and 3537 auto-agglutinated immediately after preparation. The rest of the strains except 3368 became unstable before 12 days. Strain 3368 became unstable after 18 days. Hence agglutination antigen for BAT was used within 2 weeks of preparation.

4.8.3. Exotoxin production.

All strains produced exotoxin in brain-heart infusion broth but the concentration varied greatly. Strains 3362 and 3513 produced very low concentrations of 16 MHD and 128 MHD, respectively. Table I shows concentration of exotoxin, culture pH values and quality of pellicle formation by the seven strains. A relationship was found between culture pH and the extent of pellicle formation. Cultures with thick complete pellicles tended to have high pH values than cultures with thin, partial or no pellicles. No relationship between culture pH and exotoxin concentration was evident.

4.9. Bacterial agglutination test.

Figure 8 shows the distribution of serum agglutination titres among the three sheep age groups. The titres ranged from 1/8 to 1/32,768 in adult sheep sera; 1/8 to 1/2048 in 6 months old and 1/4 to 1/16 in precolostral lambs sera. The range of observed titres increased with increase in age among

the groups with the mean titres being 1/7.4 for precolostral sera, 1/79.4 for 6 months olds and 1/158.5 for adult sheep. There was complete overlap between titres from adult sheep and 6 months old lambs and also partial overlap between titres from these two groups and those from precolostral sera. In adult sheep group, there was a distinct tailing of titres towards higher values, which was absent in precolostral and 6 months old groups.

4.10. Specificity of agglutination reaction.

Table 2 shows serum agglutination titre values before and after treatment of sera with staphylococcal protein A, C. pseudotuberculosis agglutination antigen or 2 - mercaptoethanol. After treatment of sera with any of these reagents, no significant reduction in their agglutination titres to C. pseudotuberculosis was observed.

No cross-reaction between C. pseudotuberculosis and C. pyogenes was detected in agglutination reaction. Sheep immunised with C. pyogenes did not show a rise in agglutination titre to C. pseudotuberculosis. In ELISA test, the immunised sheep showed a high response to C. pyogenes antigen. At a serum dilution of 1/10,000 the preimmune serum had an OD value of 0.044 and that of the immune serum was 0.20.

4.11. Haemolysis inhibition test titres.

Of the 1030 sera examined in HIT, only 89 had serum haemolysis inhibition titres of 1/4 and above.

At a serum dilution of 1/2, erythrocytes had a tendency to haemagglutinate and this was taken as non-specific inhibition of haemolysis. Positive titres were therefore taken as those of 1/4 and above.

All positive sera were from animals over one year old and of these, 67% were from 4-6 years old. None of the sera from precolostral lambs or 6 months old lambs had a detectable haemolysis inhibition titre.

The haemolysis inhibition test showed a high reproducibility of titres. The average standard deviation of titres in the five samples tested for reproducibility was $\pm$ 0.12.

4.12. Specificity of HIT reaction.

A sheep serum sample positive in HIT showed a reaction of identity with Rabbit anti- C. pseudotuberculosis serum against C. pseudotuberculosis exotoxin in agar gel immunodiffusion test. Figure 9 shows precipitin bands between C. pseudotuberculosis exotoxin and the two sera. At least two of the precipitin bands had a reaction of partial identity.

4.13. Comparison between BAT and HIT.

There was no correlation between BAT and HIT titres. Samples with high BAT titres did not have correspondingly high HIT titres and vice versa. Although all samples could agglutinate the antigen to varying titres, only 89 of them, representing 8.6% of the samples had positive HIT titres. However,

samples with positive HIT titres also had high BAT titres. Of the 87 samples that were positive in HIT, 74 had BAT titres equal to or above 1/512 and the rest had titres between 1/128 and 1/256. A BAT titre of 1/512 and above was taken as the cut-off point. This cut-off point excluded all precolostral lambs and 94.8% of the 6 months old lambs but included 17.7% of the adult sheep.

4.14 Anti - C. pseudotuberculosis exotoxin in the ELISA test.

The ELISA test was able to detect antibodies to C. pseudotuberculosis exotoxin in sheep sera. The ELISA OD values had a high correlation to HIT titres. Figure 10 shows the correlation between ELISA OD values and HIT titres. A correlation coefficient of 0.86 was calculated ($P < 0.001$).

The ELISA was also able to highly discriminate between samples that had the lowest HIT titres and those that were negative. In this respect, the ELISA test appeared more sensitive than HIT test.

4.15. Prevalence of C. pseudotuberculosis seropositive cases among the sheep flock.

Table 3 shows the number of BAT, HIT and both BAT and HIT seropositive animals in groups of flocks located in different districts. In most flocks there were no HIT seropositive cases. Flocks in Barum Verk had the highest prevalence followed by flocks in Konsberg.

The prevalence was very low in animals from Osterdaren and Lillehammer, but these animals were 6 months old. Two flocks examined in Tostard had a high prevalence in BAT although none of the samples was seropositive in HIT. In Aludal, sera from only one flock were examined and showed a high prevalence rate. Overall, C. pseudotuberculosis infections appeared restricted to some particular districts.

4.16. Prevalence of seropositive cases among different breeds of sheep.

Table 4 shows prevalence of C. pseudotuberculosis seropositivity among the two major breeds of sheep in the region, the Spœl and Dala breeds; a lesser breed, the Ryggja; and two crossbreeds. Samples from the Ryggja breed were too few for effective comparison and samples from the two cross-breeds, the Dala x Finnsau and Dala x Spœl, were from 6 months old slaughter lambs. Among the two major breeds, the Dala and spœl, the spœl appeared to have a higher prevalence rate than the Dala breed.

4.17. Effect of Age on the prevalence of C. pseudotuberculosis seropositivity in sheep.

Table 5 shows the distribution of seropositive cases among the different age groups of sheep examined. The highest prevalence was found in animals of 5 - 6 year old. There was a high correlation between age and prevalence in either BAT, HIT or in both tests. Correlation

coefficients were 0.96 for BAT, 0.78 for HIT and 0.78 for both tests ($P < 0.001$). The prevalence appeared to decline in animals 7 years old and above.

5. DISCUSSION.

Metabolites of C. pseudotuberculosis and C. equi interacted to cause complete lysis of sheep or ox erythrocytes but none of them could effect lysis alone. The activity of each was inhibited by the homologous antiserum and was therefore specific. Under the experimental conditions used, erythrocyte lysis was therefore dependent on both substances.

Sheep erythrocytes pre-treated with C. pseudotuberculosis exotoxin were more rapidly haemolysed when exposed to C. equi metabolite than were erythrocytes pre-treated with C. equi metabolite and then exposed to C. pseudotuberculosis exotoxin(Fig. I). This shows that lysis of erythrocytes was initiated by C. pseudotuberculosis exotoxin and completed by C. equi metabolite. This was in contrast to the belief hitherto put forward that in this haemolytic system, C. equi metabolite had the role of an erythrocyte sensitizer for lysis by C. pseudotuberculosis exotoxin (Knight, 1978). In the present study, erythrocyte lysis appear to take place in two stages. The first was effected by C. pseudotuberculosis exotoxin and is independent of C. equi metabolite. The second was effected by C. equi metabolite and was dependent on prior activity of C. pseudotuberculosis exotoxin on erythrocytes. Whereas these conclusions were independently arrived at from

results of the investigation presented in the present work. It was later found out that Bernheimer et al. (1980), working on activity of corynebacterial phospholipases on membrane sphingomyelins had arrived at a similar conclusion. They found that haemolysis of sheep erythrocytes was accomplished by sequential activity of C. pseudotuberculosis phospholipase D and an extra-cellular protein of C. equi in presence of magnessium ions, followed by chilling. In the present work, the phenomenon was investigated under the conditions of the haemolysis inhibition test and no additional factors were employed to effect haemolysis.

C. pseudotuberculosis exotoxin sphingomyelinase hydrolyses erythrocyte cell membrane sphingomyelins but this activity does not cause haemolysis (Souckova and Soucek, 1972). This activity however, potentiates haemolytic activity of staphylococcal delta-lysin (Lovell and Zaki, 1966) and of C. equi metabolite, as seen from the present results. The staphylococcal delta-lysin may have an activity similar to that of C. equi metabolite.

In figure 2, C. pseudotuberculosis exotoxin produced partial haemolysis in ox blood agar, an observation also made by Zaki (1965a). However, when erythrocytes were incubated in suspension with the exotoxin, no lysis was observed except for a yellow colouration appearing in the supernatant fluid. This

yellow colour may be attributed to products of sphingomyelin hydrolysis. The release of these products appear to dilute erythrocyte colour such that in blood agar they appear pale. This effect of exotoxin on erythrocytes was inhibited by antiserum to C. pseudotuberculosis but not by antiserum to C. equi metabolite. This was an indication that the exotoxin of C. pseudotuberculosis and the metabolite of C. equi do not cross-react serologically.

Mixed cultures of C. pseudotuberculosis and C. equi produced wide zones of beta-haemolysis which was inhibited by antiserum to either C. pseudotuberculosis or to C. equi metabolite (Figure 3). C. pseudotuberculosis culture alone produced a narrow zone of beta-haemolysis. This haemolysis seems to be due to activity of a substance from the organism, other than the exotoxin, since exotoxin produced only partial haemolysis in blood agar (Figure 2). Furthermore, this haemolysis was not inhibited by antiserum to C. pseudotuberculosis unlike the activity of exotoxin which was inhibited by the antibacterial serum. This supports conclusions by Carne (1939) that the haemolysin oc C. pseudotuberculosis is different from the exotoxin and that it is non-antigenic, as it is not inhibitable by anti- C. pseudotuberculosis serum.

C. equi metabolite from cultures of strain NVH 3370 was found to contain very low concentrations of the active substance. It is possible that there is strain variation in production of the substance, with strain NVH 3370 being a poor producer.

The active substance of C. equi metabolite was thought to be a protein. This follows from the fact that it was precipitated by ammonium sulphate. The activity of the substance was completely neutralised by the homologous antiserum raised in rabbits. Knight (1978) failed to demonstrate this specific inhibition of C. equi metabolite activity using sera from foals infected with C. equi. This failure may have been due to inadequate antibody concentration in the sera.

In sephadex-G 100 fractionation, C. pseudotuberculosis exotoxin activity (Figure 4), was found to spread throughout the column as also observed by Onon (1978). Onon attributed this spread to the glycoprotein nature of the exotoxin. However, definite peaks of activity could be identified in the present work. It appears therefore, more likely that the exotoxin consists of several protein aggregates of different molecular weights having the same biological activity. Such an opinion is supported by the work of Goel and Singh (1972) who demonstrated two proteins of different molecular weights in the exotoxin.

C. equi metabolite active substance was eluted homogeneously, indicating that it consists of a single protein (Figure 5). The elution volume was twice that for C. pseudotuberculosis exotoxin and therefore the substance has a much lower molecular weight. In the elution profile, the absorbance of the eluate representing the activity peak was very low. This again reflected the low concentration of the active substance in crude C. equi metabolite.

There was no evidence of serological cross-reaction between C. pseudotuberculosis exotoxin and C. equi metabolite. Antiserum to C. equi metabolite did not inhibit activity of C. pseudotuberculosis exotoxin on erythrocytes (Figure 2). In agar gel immunodiffusion, crude C. equi metabolite formed precipitin lines with antiserum to C. pseudotuberculosis but were not identical to those formed by C. pseudotuberculosis exotoxin with antiserum to C. pseudotuberculosis (Figure 6). However, sephadex-purified active C. equi substance formed no bands with antiserum to either C. pseudotuberculosis or to C. equi metabolite. The reaction between crude C. equi metabolite and C. pseudotuberculosis antiserum must therefore be a cross-reaction between a different substance in C. equi metabolite and an antigen of C. pseudotuberculosis other than the exotoxin. Antiserum to C. equi metabolite formed a precipitin band with

either C. pseudotuberculosis exotoxin (Figure 7) or crude C. equi metabolite. The precipitin band between antiserum to C. equi metabolite and C. pseudotuberculosis exotoxin was however not identical to that between C. pseudotuberculosis antiserum and C. pseudotuberculosis exotoxin and therefore involved different antibodies and antigens. The reaction between antiserum to C. equi metabolite and C. pseudotuberculosis exotoxin most probably involves antibodies raised against brain-heart infusion proteins present in the crude C. equi metabolite inoculated into the rabbits during immunisation process. These proteins are also present in C. pseudotuberculosis exotoxin. This reaction is not therefore unexpected.

It had been suspected that either the product of C. equi which together with C. pseudotuberculosis exotoxin caused lysis of erythrocytes, or another product of C. equi may have a morphological similarity to C. pseudotuberculosis exotoxin which would then mean antigenic cross-reaction. This was in view of the observation that crude C. equi metabolite interfered with the activity of C. pseudotuberculosis exotoxin. In figure I, erythrocytes pre-treated with C. equi metabolite had a longer haemolysis time than the controls. It was thought possible that a substance in C. equi metabolite with a molecular configuration similar to that of C. pseudotuberculosis exotoxin

may have bound the substrate making it unavailable for C. pseudotuberculosis sphingomyelinase. This would now be plausible in view of findings by Bernheimer et al. (1980) that a C. equi factor also splits sphingomyelins. However, no cross-reaction could be demonstrated in either neutralisation test (Figure 2) or in agar gel immunodiffusion tests. The interference could therefore be either non-specific or the antigenic determinant sites may be different from substrate binding sites. As such, C. equi metabolite may bind substrate for but not antibodies against C. pseudotuberculosis exotoxin.

Agglutination antigen prepared with the use of Tween 80 from an attenuated strain of C. pseudotuberculosis, was found stable enough for use in BAT. The spontaneous agglutination of C. pseudotuberculosis cells is caused by adherence of the wax-like surface lipid (Jolly, 1966; Hard 1969). Repeated subculturing of C. pseudotuberculosis strain NVH 2586 diminished tendency to autoagglutinate. This might have been due to a reduction in amounts of the lipid substance. Jolly (1966) and Hard (1969) found less amount of the lipid substance in attenuated than in pathogenic strains. However, Tween 80 did not prevent autoagglutination of most of the non-attenuated strains. Jolly (1966) similarly found that the detergent did not inhibit pellicle formation by non-attenuated strains.

Strain NVH 3368 was non-haemolytic and of low exotoxigenicity but the original strain, NVH 2586, was haemolytic and highly exotoxigenic although the biochemical activity of both strains were identical. This was in agreement with observations by Barakat et al. (1970) that repeated subculturing transforms haemolytic C. pseudotuberculosis strains to non-haemolytic, and by Burrell (1979 ~~1970~~) that exotoxigenicity is reduced with repeated subculturing. That strain NVH 3368 was non-haemolytic but still produced a detectable amount of exotoxin was further evidence that the haemolysin and the exotoxin of C. pseudotuberculosis are different substances.

In table 1, C. pseudotuberculosis culture pH appeared to be influenced by aeration. Bacterial cells in cultures with pellicles would receive more oxygen at the surface than precipitated bacterial cells. Such cultures with pellicles tended to have higher pH values than cultures with little or no pellicles. However, no relationship between exotoxin, concentration and culture pH was evident. Doty et al. (1964) had observed maximum exotoxin concentration to coincide with a certain culture pH value.

Sheep sera was found to agglutinate C. pseudotuberculosis non-specifically since the agglutination was not affected by treatment of sera with either staphylococcal protein A which would bind

IgG, 2-mercaptoethanol which would denature IgM, or by adsorption with C. pseudotuberculosis cells which would adsorb any specific antibodies to the bacterial cell surface. Burrell (1978) also found that precolostral lamb sera agglutinated C. pseudotuberculosis non-specifically. A non-specific factor has been found in bovine sera which agglutinates Brucella abortus (Amerault et al., 1961; Rose et al., 1964). However unlike the sheep serum factor, the bovine serum factor could be adsorbed by Brucella organisms (Rose et al., 1964). The non-specific sheep serum factor therefore appears to be a normal constituent of the serum. The concentration of the factor apparently increases with the age of the animal since it was low in precolostral but generally high in adult sheep sera. The distribution of BAT titres among the adult sheep, with a small fraction having outstandingly high titres (Fig. 6) also suggests that the concentration of the factor in serum may be enhanced by C. pseudotuberculosis infection. Furthermore, it has been found that antibodies to C. pseudotuberculosis cell surface are not easily produced (Cameron and Fuls, 1973; Shigidi, 1974) probably due to the intracellular location of the parasite.

A factor in sheep sera able to agglutinate C. pseudotuberculosis non-specifically would have important implications. Firstly the BAT would be an

inaccurate diagnostic test. Secondly it may explain the normally limited spread of the organism in natural infections in sheep. In guinea pig experimental infection, Carne et al. (1956) found the organism to spread extensively, resulting in extensive pyogenic lesions in internal organs but not so in sheep. A clumping factor would control such spreading although the exotoxin from the organism may act as a spreading factor (Jolly, 1965).

Unlike BAT, a positive HIT reaction is a specific indication of antibodies to C. pseudotuberculosis exotoxin. A sheep serum sample positive in HIT was found to have a reaction of identity to rabbit antiserum to C. pseudotuberculosis when tested against C. pseudotuberculosis exotoxin in agar gel immunodiffusion (Figure 9). HIT in sheep therefore detected the same antibodies as those raised in rabbits immunised with live C. pseudotuberculosis. Furthermore, rabbit anti-C. pseudotuberculosis serum neutralised activity of exotoxin but normal rabbit did not. Exotoxins from different strains of C. pseudotuberculosis are also antigenically identical (Doty et al., 1964., Lovell and Zaki 1966). HIT is therefore a more accurate test than BAT and a positive HIT reaction is a definite indication of infection. Although non-exotoxigenic strains of C. pseudotuberculosis have been isolated from other species, (Lovell and Zaki, 1966) which would

mean possible presence of infection in absence of antibodies to exotoxin, none has been recovered from sheep. On the contrary, attenuated non-exotoxigenic strains have been found to regain their exotoxigenicity when passaged through sheep (Burrell, 1979).

A BAT titre of 512 or more was considered positive. This was the titre that most closely corresponded with HIT positive cases. Of the 89 HIT positive samples, 74 had BAT titres of 512 or more and the rest had titres of between 128 and 512. Also as it was be seen from figure 8, a BAT cut-off point of 512 and above excluded all precolostral and almost all of the 6 months old lambs. Lund et al. (1982 a) using the same test in a known infected goat herd considered a titre of 64 or more as seropositive. A cut-off titre of 64 here would include most of the young lambs which are not expected to be infected.

In animals with a BAT titre of 512 or more, only less than a half were positive in HIT (Table 3). This may be due to a high concentration of the non-specific agglutinating factor in some animals in absence of infection. Although antibodies to C. pseudotuberculosis exotoxin in infected goats have been found to diminish with time (Lund, 1983) this is unlikely to account for large difference between BAT positive and HIT negative samples.

C. pseudotuberculosis did not cross-react with C. pyogenes by agglutination reaction. Shigidi (1974) similarly found no cross-reaction by agglutination tests using antigens prepared differently from agglutination antigen used in the present work, and Shen et al. (1982) found no cross-reaction in ELISA. C. pyogenes is an enviromental contaminant and can also cause lesions in lymphnodes (Richard et al., 1979) thus requiring consideration as a differential diagnosis for caseous lymphadenitis.

In table 3, of the samples examined by both BAT and HIT, 18.3% were positive in BAT, 8.6% positive in HIT and 7.2% were positive in both tests. These values are much lower than those found by workers elsewhere. Awad (1960), in a survey found a 33% infection rate. Lund et al. (1982a) obtained a seropositivity rate above 90% in an infected goat herd. In the sheep flocks examined in this work, infection appears limited to a few flocks where it has a high prevalence. This could mean that there is little transfer of animals from one flock to another, which minimises chances of spread from flock to flock.

A high correlation was found between age and incidence of seropositivity (Table 5). This was in agreement with findings by Awad et al. (1977b) in sheep and by Ashfaq and Campbell (1979) and Lund et al. (1982a) in goats.

The data on effect of breed on prevalence of seropositivity was inconclusive. In table 4 the

Spoel breed appear to have the highest prevalence. However, the highest number of samples examined were from the Spoel breed. The cross-breeds were all young lambs and so comparison was not possible and samples from the Ryggja breed were too few. No outright breed predisposition has been found by others.

An ELISA test for antibodies to C. pseudotuberculosis exotoxin was found feasible. Such a test would be easier to perform than that performed with other antigens (Shen et al., 1982) since the exotoxin would be easier to prepare. Also it is evident that the exotoxin need not be purified for use. In figure 10, it is evident that the ELISA had a high correlation to HIT. Also the ability of the ELISA to highly discriminate between precolostral sera and those with lowest detectable titre in HIT makes it more sensitive than HIT. It would therefore be possible to substitute the ELISA for HIT especially where a large number of samples is involved. The ELISA would however, have to be standardised by a few HIT-tested samples.

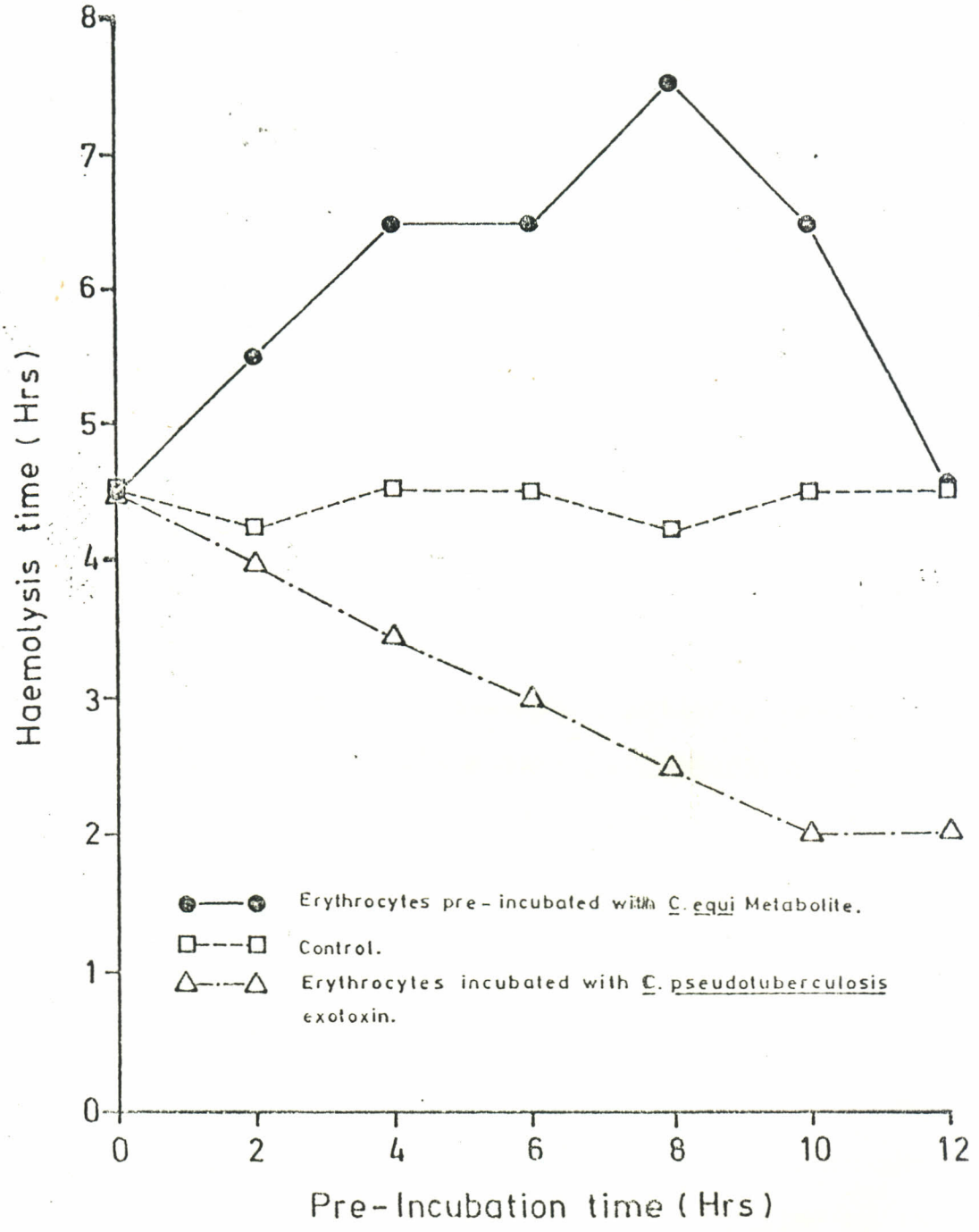


Fig.1. The effect on haemolysis time of preincubating sheep erythrocytes with either C. pseudotuberculosis exotoxin or C. equi metabolite before exposure to the other.

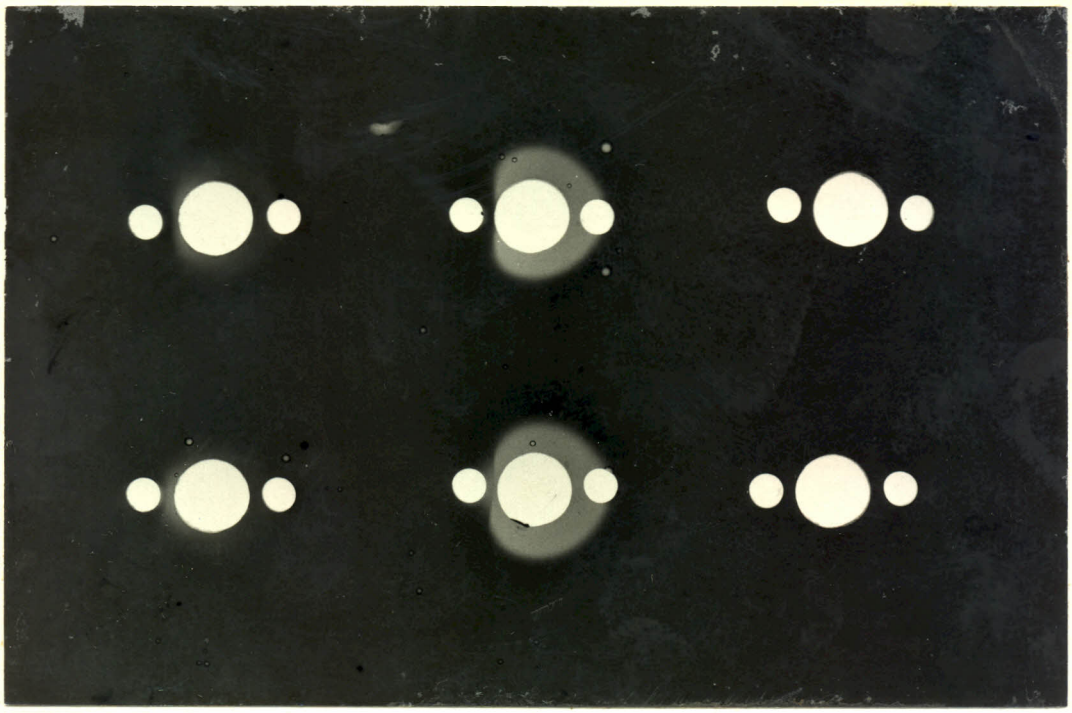


Figure 2. Neutralisation of C. pseudotuberculosis exotoxin and C. equi metabolite activity in ox blood agar by specific antisera.

Top left. C. pseudotuberculosis exotoxin (large well) partial haemolytic activity neutralised by rabbit anti - C. pseudotuberculosis serum (left) but not by normal rabbit serum (right).

Bottom left. Partial haemolysis by C. pseudotuberculosis exotoxin (large well) not inhibited by rabbit anti - C. equi metabolite (left) or normal rabbit serum (right).

Centre top. Beta haemolytic activity by a mixture of C. pseudotuberculosis exotoxin and C. equi metabolite (large well) inhited by anti - C. pseudotuberculosis antiserum (left) but not by normal rabbit serum (right).

Figure 2 Contd.

Centre bottom. Beta haemolysis by a mixture of *C. pseudotuberculosis* exotoxin and *C. equi* metabolite (large well) partially inhibited by rabbit anti *C. equi* metabolite antiserum (left) but not by normal rabbit serum (right).

Right top and bottom. No haemolysis by *C. equi* metabolite alone (large well).

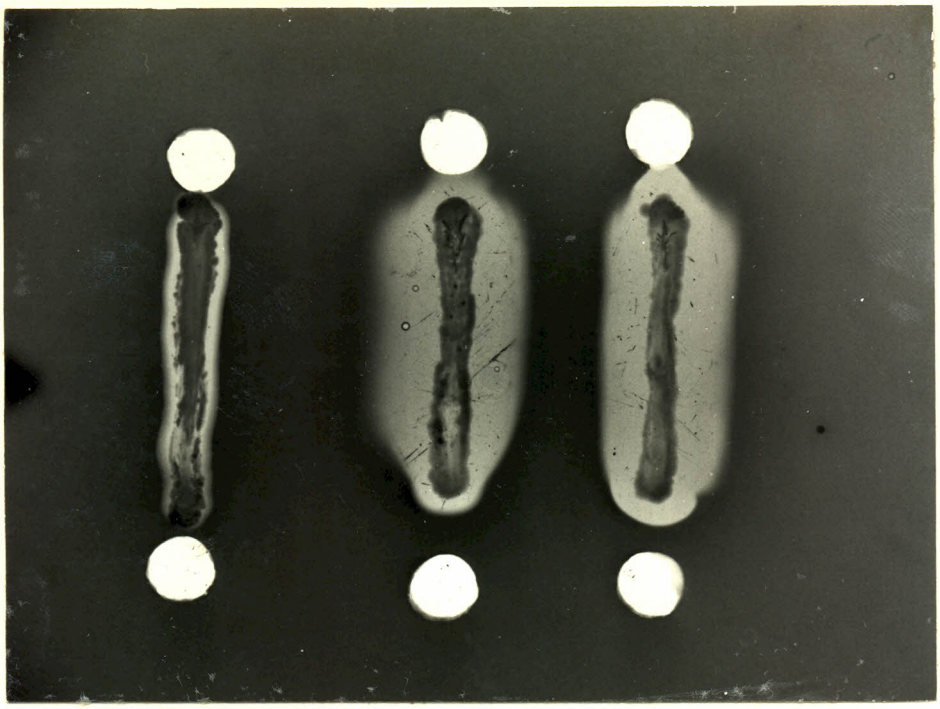


Figure 3. Neutralisation of haemolytic activity of C. pseudotuberculosis and C. equi cultures on ox blood agar.

Left. C. pseudotuberculosis culture with a narrow zone of beta haemolysis. No neutralisation of the haemolysis by rabbit anti - C. pseudotuberculosis antiserum (bottom) or normal rabbit serum (top).

Centre. Mixed culture of C. pseudotuberculosis and C. equi with a wide zone of beta haemolysis. Haemolysis inhibited by rabbit anti - C. pseudotuberculosis antiserum (bottom) but not by normal rabbit serum (top).

Right. Mixed culture of C. pseudotuberculosis and C. equi with partial inhibition of haemolysis by rabbit anti - C. equi metabolite (bottom) but not by normal rabbit serum (top).

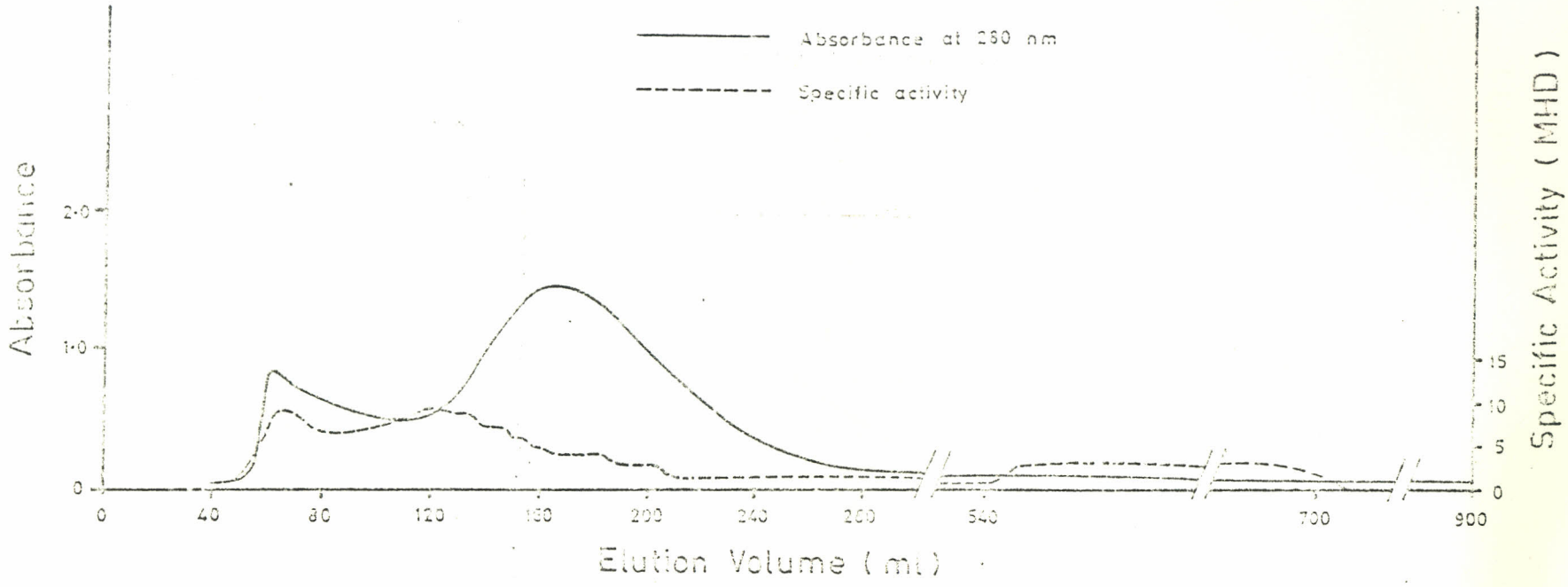


Fig.4. Sephadex G-100 elution profile of C. pseudotuberculosis exotoxin fraction $_{p}35$ - 65.

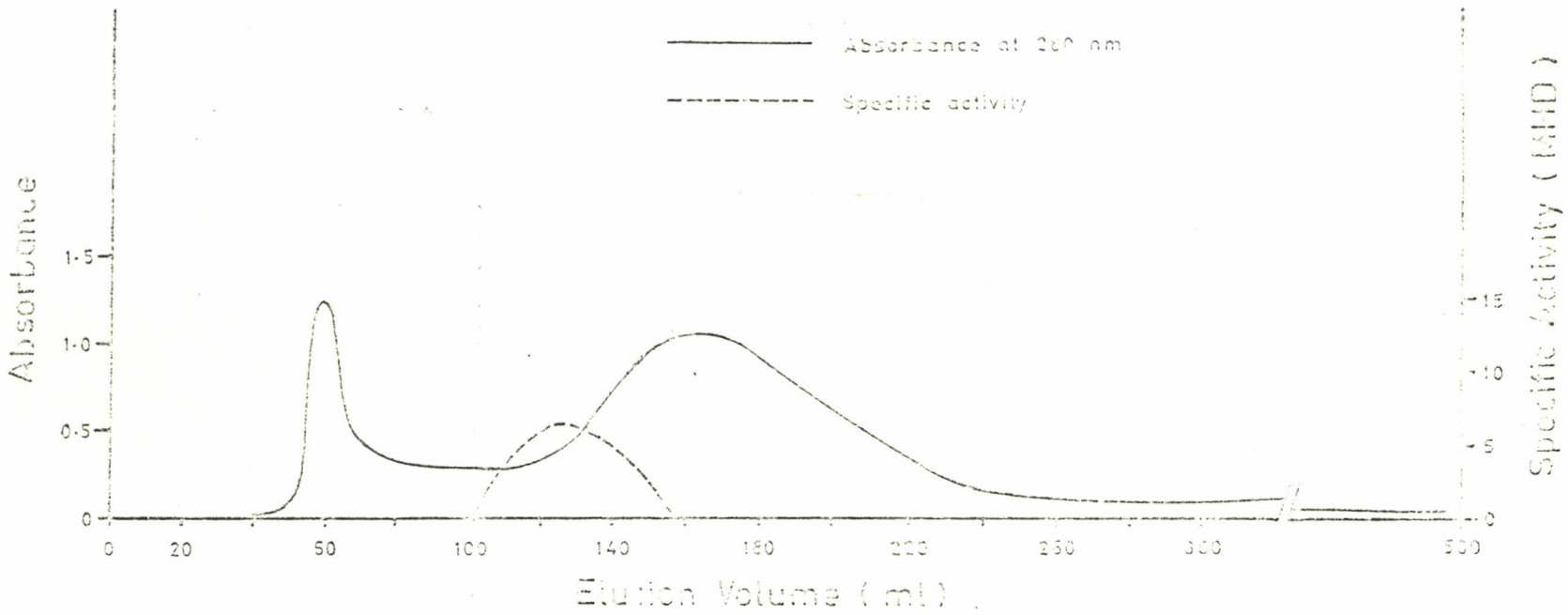
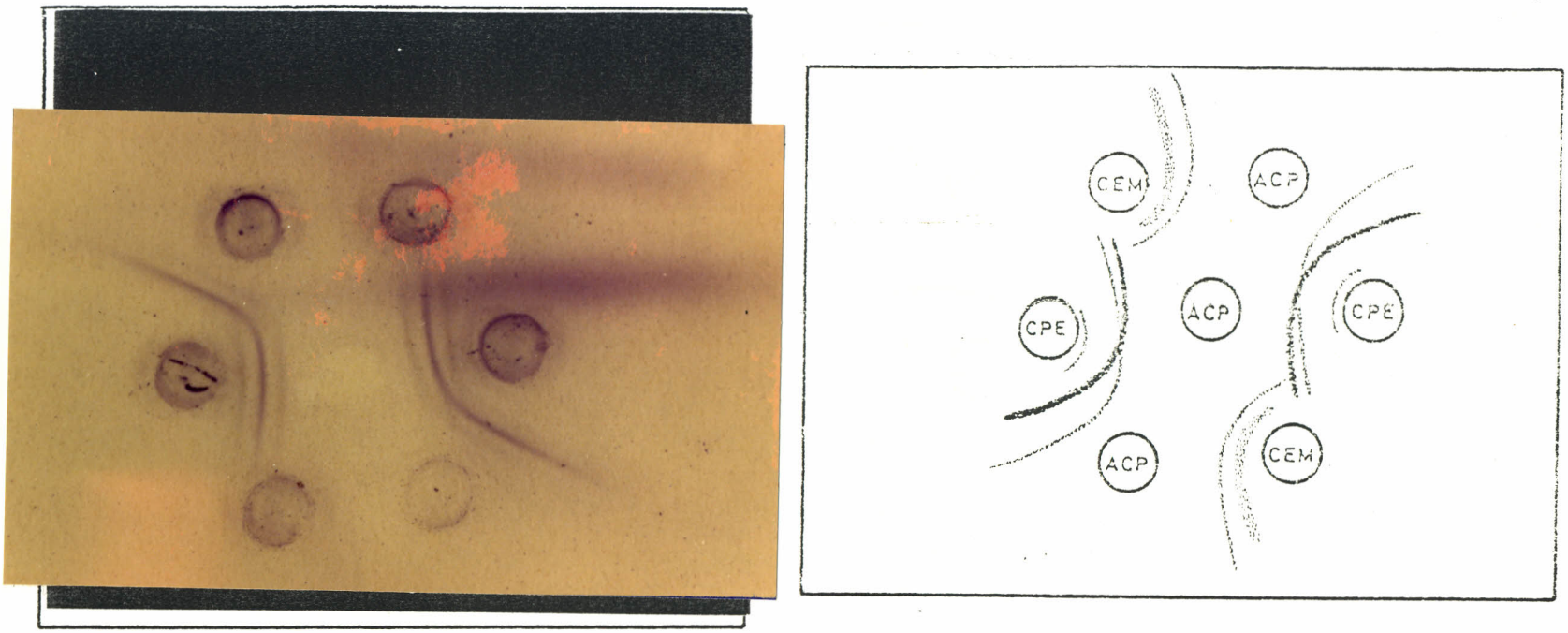
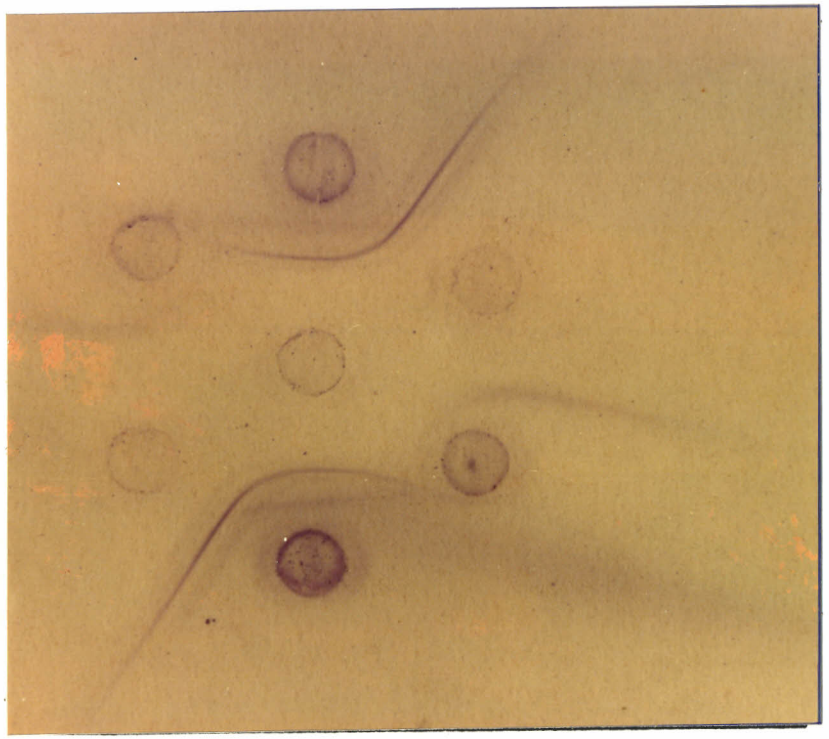


Fig.5. Sephadex G-100 elution profile of C. equi metabolite fraction p^{35} - 65.

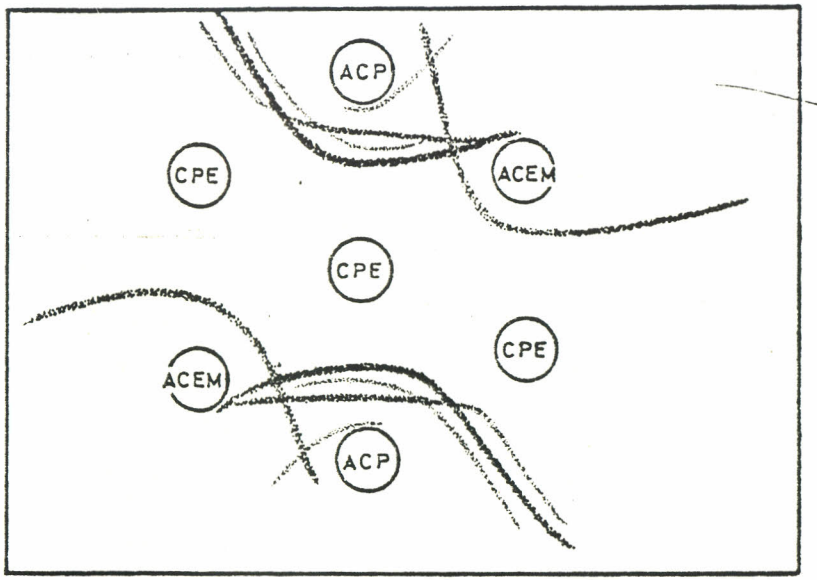


A B

Fig. 6. Photograph (A) and diagram (B) showing precipitin bands in agar gel immunodiffusion between rabbit anti - C. pseudotuberculosis antisera (ACP) and C. pseudotuberculosis exotoxin (CPE) and between rabbit anti - C. pseudotuberculosis antiserum and C. equi metabolite (CEM).



A



B

Fig. 7. Photograph (A) and diagram (B) showing reaction of non-identity of anti - C. pseudotuberculosis antiserum (ACP) and anti - C. equi metabolite antiserum (ACEM) to C. pseudotuberculosis exotoxin (CPE) in agar gel immunodiffusion.

Table 1. Production of exotoxin, pellicle formation and pH values in cultures of seven C. pseudotuberculosis strains.

Strain	Exotoxin concentration in MHD per 50 ul.	Culture pH	Pellicle quality
2586	32,768	6.59	P
3368	16	8.10	C
3381	65,536	8.21	C
3513	128	5.45	N
3534	over 130,000	8.30	C
8537	over 130,000	7.45	P
3452	65,536	8.10	C

Pellicle quality

P = partial

C = complete

N = none.

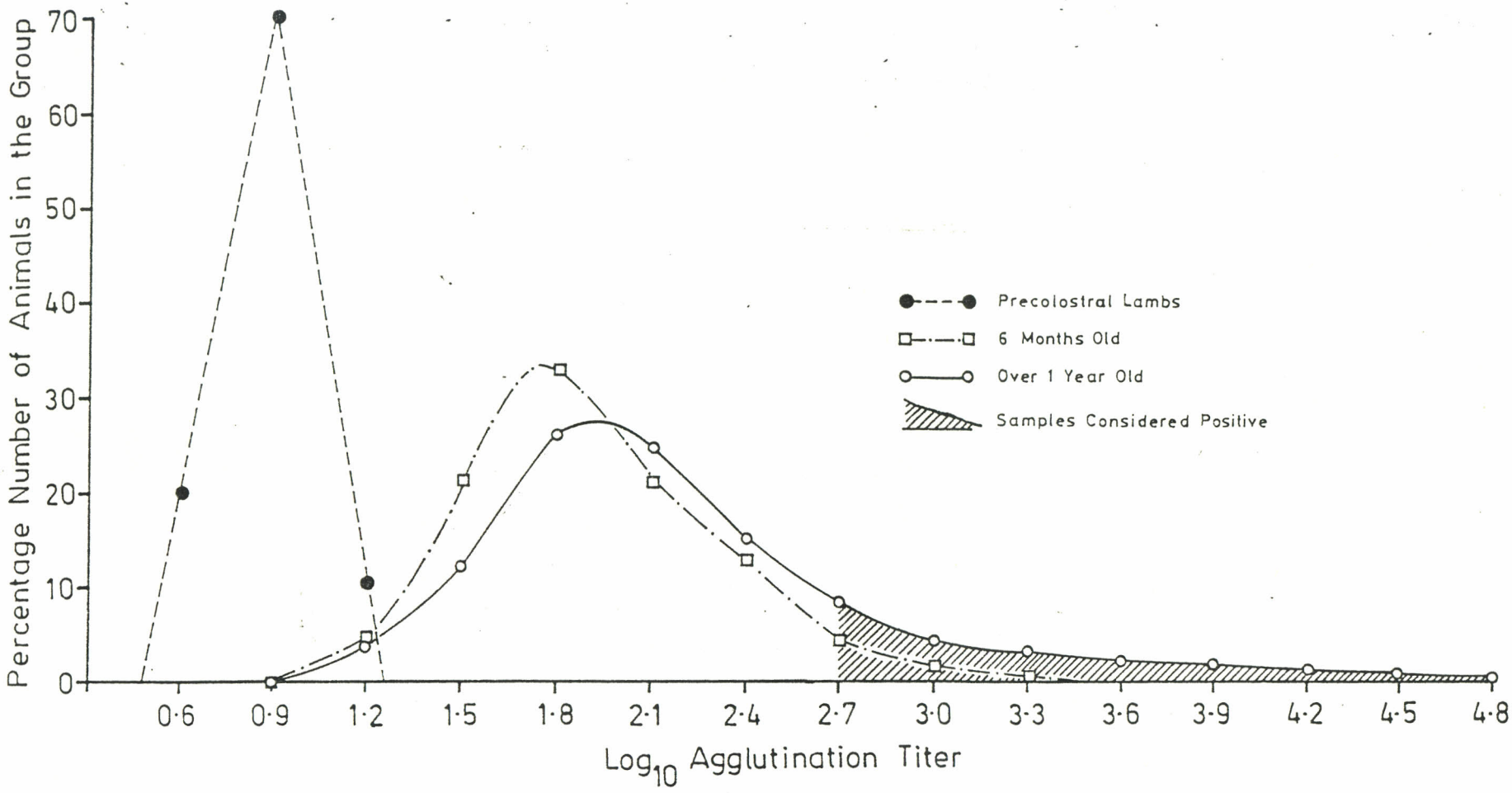
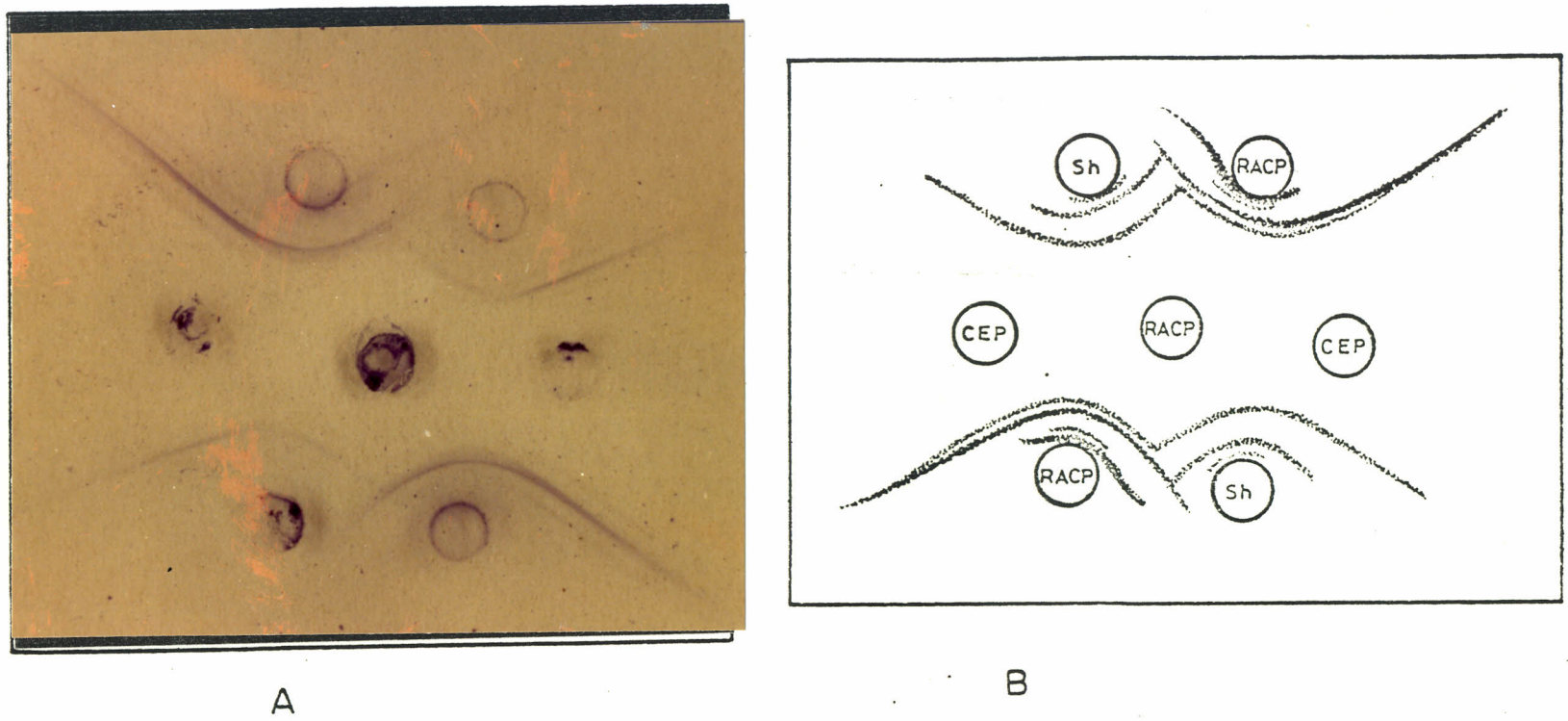


Fig. 8. Bacterial agglutination titres of sera from three age groups of sheep.

Table 2. Agglutination titres for C. pseudotuberculosis by sheep sera before and after treatment of sera with Staphylococcal protein A, C. pseudotuberculosis agglutination antigen and 2-Mercaptoethanol.

Treatment	Serum samples tested			
I. Staphylococcal protein A	5070	2023	2024	Precolostral
Test	512	16	16	8
Control	1024	16	16	8
II. C. pseudotuberculosis Agglutination antigen				
Test	64	8	8	4
Control	128	8	8	4
III. 2-Mercaproethanol				
Test	256	8	8	4
Control	256	8	8	4



A B

Fig.9. Photograph (A) and diagram (B) showing reactions of identity of rabbit anti - C. pseudotuberculosis antiserum (RACP) and a HIT - positive sheep serum (Sh) to C. pseudotuberculosis exotoxin (CEP).

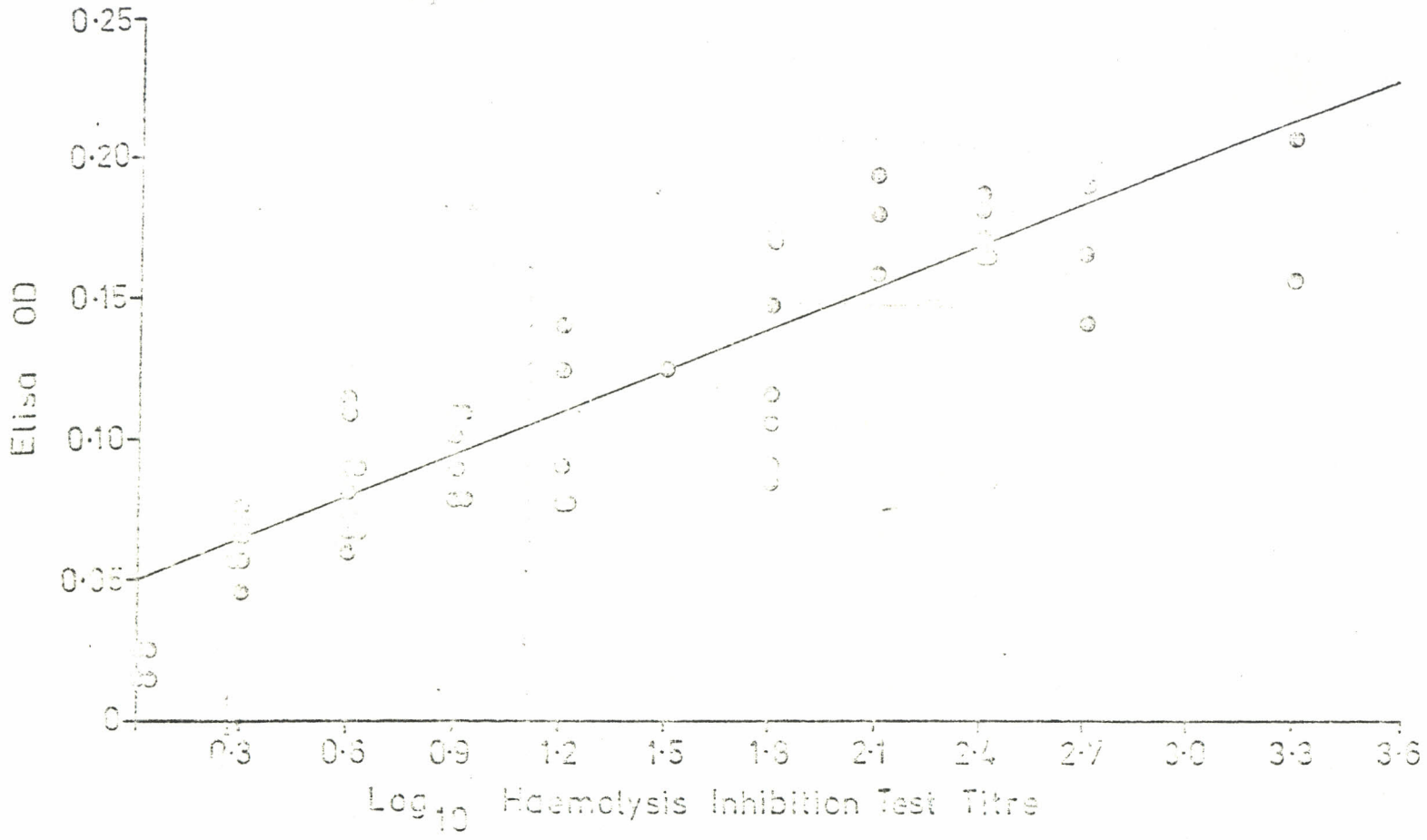


Fig.10. Comparison between HIT and anti - C. pseudotuberculosis exotoxin ELISA test. y = 0.05 + 0.05 x.

The correlation is 0.86.

Table 3. Prevalence of C. pseudotuberculosis seropositive cases among groups of sheep flocks.

Locality of flocks	Number Examined	BAT Positive	BAT %	HIT Positive	HIT %	BAT AND HIT Positive	BAT AND HIT %
Barum verk	80	58	72.5	58	72.5	49	61.3
*NLH	50	11	22.0	12	24.0	7	14.0
+NVH	22	11	50.0	8	36.4	8	36.4
Lommedalen	30	2	6.6	0	0.0	0	0.0
Fyresdal	295	37	12.5	0	0.0	0	0.0
Hauggrend	60	8	13.3	0	0.0	0	0.0
Tostard	17	12	70.5	0	0.0	0	0.0
Aludal	11	7	63.6	2	18.2	2	18.2
Vikelund	21	3	14.2	0	0.0	0	0.0
Urdal	66	2	3.0	0	0.0	0	0.0
Konsberg	33	15	45.5	9	27.3	8	24.2
Geilo	24	2	8.3	0	0.0	0	0.0
Kverualand	15	1	6.6	0	0.0	0	0.0
Qsterdalen	197	14	0.7	0	0.0	0	0.0
Lillehammer	109	6	0.6	0	0.0	0	0.0
	1030	189	18.3	89	8.6	74	7.2

* = Norwegian College of Agriculture.

+ = Samples from Norwegian Veterinary College, department of Parasitology.

Table 4. Prevalence of C. pseudotuberculosis seropositive cases among different sheep breeds.

Breed	Number	BAT		HIT		BAT AND HIT	
	Examined	Positive	%	Positive	%	Positive	%
Spoel	428	103	24.0	58	13.6	49	11.4
Dala	295	36	12.2	21	7.1	15	5.1
Ryggja	22	11	50.0	8	36.4	8	36.4
Dala X Finasau	82	2	2.4	0	0.0	0	0.0
Dala X Spoel	115	12	10.4	0	0.0	0	0.0
Not known	88	25	28.4	2	2.3	2	2.3

Table 5. Effect of age on the prevalence of *C. pseudotuberculosis* seropositive cases in sheep.

Age in years	Number Examined	BAT Positive	BAT %	HIT Positive	HIT %	BAT AND HIT Positive	BAT AND HIT %
0	10	0.0	0.0	0	0.0	0	0.0
0.5	274	16	5.8	0	0.0	0	0.0
1	88	7	8.0	1	1.1	1	1.4
2	100	19	19.0	12	12.0	10	10.0
3	72	19	26.4	9	12.5	8	11.1
4	67	20	29.9	16	23.9	12	17.9
5	49	20	40.8	16	32.7	13	26.5
6	32	18	56.3	15	46.9	14	43.8
7 and over	36	18	50.0	8	22.2	8	22.2

6. CONCLUSIONS.

The following were the major conclusions arising from this work.

(1). The lysis of erythrocytes by C. pseudotuberculosis exotoxin and C. equi metabolite, as it occurs in the HIT is effected by C. equi metabolite subsequent to prior activity of C. pseudotuberculosis exotoxin on the erythrocytes. C. equi metabolite does not act as sensitizer.

(2). No erythrocyte lysis takes place in absence of either substance or in presence of antiserum to either. The HIT test can therefore not only be used to detect antibodies to C. pseudotuberculosis exotoxin in C. pseudotuberculosis-infected animals but also to detect antibodies to C. equi metabolite in C. equi infected animals.

(3) C. pseudotuberculosis exotoxin is composed of several proteins of different molecular weights but the active substance in C. equi metabolite consists of a single heat-labile protein. In addition to exotoxin production, C. pseudotuberculosis produces a haemolysin that is not detectable in culture filtrate and is apparently non-antigenic.

(4). Although erythrocytes pre-exposed to C. equi metabolite appear to be altered in such a way that they are less readily affected by C. pseudotuberculosis exotoxin, the two substances do not cross-react serologically. However, a substance in C. equi

metabolite cross-reacts with another antigen of C. pseudotuberculosis bacterium.

(5) In bacterial agglutination test (BAT) for antibodies to C. pseudotuberculosis, stable agglutination antigens can be obtained from strains attentuated by passage through artificial media. However, agglutination of C. pseudotuberculosis by sheep sera is non-specific. This makes BAT an inaccurate test for detecting C. pseudotuberculosis infections in sheep. The ability of sheep sera to agglutinate the organism appear to increase with age and possibly with infection by the organism. Infection by C. pyogenes does not contribute to this agglutination.

(6) The haemolysis inhibition test (HIT) for detection of antitoxin to C. pseudotuberculosis exotoxin is a specific and a highly reproducible test but can be effectively substituted with enzyme-linked immunosorbent assay (ELISA). The ELISA would have the advantage of rapidity and capacity for testing a large number of samples.

(7) C. pseudotuberculosis infections in sheep in southern Norway are not widespread but confined to some particular flocks. No breed predisposition is apparent but a high positive correlation exists between age of animals and the prevalence of seropositivity.

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