

**A STUDY OF THE EPIDEMIOLOGY AND CONTROL OF
CORYNBACTERIUM PSEUDOTUBERCULOSIS
INFECTION OF SHEEP IN THE UNITED KINGDOM**

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ABSTRACT

This thesis is a distillation of work carried out by the author and others between 1999 and 2010. The overarching objective was to achieve a better understanding of the epidemiology and prevalence of ovine caseous lymphadenitis within the UK sheep industry. Using that knowledge, means by which the disease could be controlled were then evaluated. Throughout this process the existing knowledge of *Corynebacterium pseudotuberculosis* and the diseases that it causes, provided a foundation on which these further developments were built.

Although *C. pseudotuberculosis* infection has been recognised in the UK for a comparatively short period, it has been a cause of significant disease in various animal species around the globe for many decades. A comprehensive review of *C. pseudotuberculosis* disease and related research was therefore an essential starting point for consideration of the infection in this country.

The use of phospholipase D ELISA tests has been an established tool of CLA research in other countries. Using a recently developed ELISA assay and blood samples collected from around GB for the Sheep Health Scheme, the prevalence of CLA within the terminal sire sector of the British sheep industry was assessed. Seropositive animals were detected in 18% of the 745 flocks tested, with the Charollais breed showing the highest apparent prevalence.

In response to concerns within the dairy goat sector regarding milk hygiene in CLA affected herds, the ability of normal commercial pasteurisation techniques to kill *C. pseudotuberculosis* organisms in milk was established.

The potential of using CLA ELISA and Western blot assays to control and eradicate the disease was studied: first in a number of lowground terminal sire flocks and then in a

large upland commercial flock. In both situations a policy of test and cull was shown to be effective in substantially reducing clinical manifestations of disease. Complete eradication was found to be achievable under certain circumstances. In a lowground flock where initial seroprevalence was greater than 60%, a complete absence of clinical disease and a seronegative status was achieved. In a large upland flock, seroprevalence was reduced from 10% to 0.4%. More significantly, the incidence of clinical CLA in this flock, which in 2007 was approximately 5% amongst the adult animals, was reduced to zero for a period of two years from June 2008.

Vaccination, which forms the mainstay of CLA control efforts in many parts of the world, was assessed for its potential for disease control in the UK. The market-leading proprietary vaccine was compared with three experimental vaccines in its ability to confer protection against bacterial challenge. A recombinant PLD and bacterin combined vaccine was shown to confer the most effective protection.

KEY WORDS

***Corynebacterium pseudotuberculosis*, caseous lymphadenitis,
sheep, United Kingdom**

STATEMENT OF ORIGINALITY

This thesis has been composed by the candidate alone. The work described herein is the author's own. Where work was conducted in collaboration with colleagues in a research group, the contribution of others to that work is clearly indicated and acknowledged. The work described in this thesis has not been submitted for any other degree or professional qualification. The clinical work and data collection has been conducted in accordance with the European Union Guidelines for Good Clinical Practice for the conduct of clinical trials of Veterinary Medicinal Products (GCPV) (Anon 1994).

Graham J. Baird

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Chapter 2: This section is based upon a published review (Baird and Fontaine 2007).

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Glossary:

Term	Definition
C ₁₄	Description of fatty acid chains
cfu	Colony forming units
CLA	Caseous lymphadenitis
DNA	Deoxyribonucleic acid
g	Standard gravitational force
IgG	Immunoglobulin G
IgM	Immunoglobulin M
M	Molar
mM	Millimolar (10 ⁻³ molar)
OD _{450nm}	Optical density measure at specified wavelength (in this case 450nm)
PCR	Polymerase chain reaction
PLD	Phospholipase D
rPLD	Recombinant phospholipase D
rpm	Revolutions per minute
SAC VS	Scottish Veterinary College Veterinary Services
v/v	Volume by volume
w/v	Weight by volume

Publications arising from this work (in chronological order):

Refereed publications

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Malone, F. E., Fee, S. A., Kamp, E. M., King, D. C., Baird, G. J. & Murdock, F. E. A. (2002) Postmortem and serological examination of sheep in three flocks affected with caseous lymphadenitis. In Proceedings of the Sheep Veterinary Society. Ed M. Clarkson. Malvern, Sheep Veterinary Society. pp 55-58

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* Baird, G. J., Synge, B. & Dercksen, D. (2004) Survey of caseous lymphadenitis seroprevalence in British terminal sire sheep breeds. *Veterinary Record* **154**, 505-506

* Fontaine, M. C., Baird, G. J., Connor, K. M., Rudge, K., Sales, J. & Donachie, W. (2006) Vaccination confers significant protection of sheep against infection with a virulent United Kingdom strain of *Corynebacterium pseudotuberculosis*. *Vaccine* **24**, 5986-5996

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* Baird, G. J. & Fontaine, M. C. (2007) *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. *Journal of Comparative Pathology* **137**, 179-210

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* Baird, G. J. & Malone, F. E. (2010) Control of caseous lymphadenitis in six sheep flocks using clinical examination and regular ELISA testing. *Veterinary Record* **166**, 358-362

(* Published papers are included in Appendix 1. Publisher's permission was granted for the reprinting where required.)

Other publications

Baird, G. J. (1997) Caseous lymphadenitis: An increasing cause for concern. *Veterinary Record* **140**, 611

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Malone, F. E., Fee, S. A., Kamp, E. M., King, D. C., Baird, G. J. & Murdock, F. E. A. (2002) Postmortem and serological examination of sheep in three flocks affected with caseous lymphadenitis. In *Proceedings of the Sheep Veterinary Society*. Ed M. Clarkson. Malvern, Sheep Veterinary Society. pp 55-58

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Chapter 1: INTRODUCTION TO THIS THESIS

When the *Corynebacterium pseudotuberculosis* bacterium was identified in a laboratory at the Cambridge University Veterinary School in January 1990, it was the first time that the organism had been isolated from a clinically diseased animal from the UK (Lloyd and others 1990b). While the detection of the organism was noteworthy as a bacteriological finding, it also represented the first case in this country of a disease, caseous lymphadenitis, which has troubled sheep producers in the rest of the world for more than a century. During the subsequent twenty years the condition has spread steadily within the country and at different times has become a cause for concern for producers in different sectors of the sheep industry.

This thesis presents the main areas of work carried out by the author on ovine caseous lymphadenitis. In each phase of research the work has been progressed in close collaboration with colleagues from other scientific institutes. The contribution of these colleagues is fully described in the Acknowledgement section of this thesis. Where the results of the research have been published, this too is acknowledged.

With no previous experience of CLA in the UK, a close examination of the existing scientific literature on the disease forms the starting point for any study of the disease. While this review of current literature is done principally in Chapter 2, further papers of relevance are cited in subsequent chapters.

Chapters 3 to 7 describe the research work pursued by the author. Chapter 3 describes an early study of CLA seroprevalence within Great Britain. Chapter 4 records research on the protective effects of pasteurisation in relation to the potential contamination of milk by *C. pseudotuberculosis*, a concern at the time amongst dairy goat producers. Chapters 5 and 6 review the development of a CLA control programme based on serological testing and clinical examination. This model was applied in two distinct sectors of the sheep industry: intensive low ground systems for the production of

terminal sire rams and an extensive upland commercial flock, where the sale of store lambs and female replacement stock forms the principal source of income. Chapter 7 describes a series of experiments where an artificial infection model was used to compare the protective effects of a proprietary CLA vaccine with a series of experimental vaccines developed employing a UK strain of the organism. Finally, Chapter 8 is a discussion section, where the preceding chapters are set in the context of current disease trends and the author seeks to establish the lessons gained from the last twenty years.

Chapter 2: THE VETERINARY SIGNIFICANCE OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS*

The perceived importance of caseous lymphadenitis (CLA) as a disease of sheep varies greatly around the world. In the UK, where this disease appeared for the first time in the early 1990's, the steady and apparently relentless spread of infection in sheep and goats has prompted considerable concern amongst farmers and veterinary surgeons. However, in the sheep industries of Australia and New Zealand, CLA is regarded as relatively minor infection, albeit one that is very widespread. The roots of these contrasting attitudes lie in differences in disease prevalence, available means of control and meat inspection practices.

To the veterinary pathologist, the causative organism of caseous lymphadenitis, *Corynebacterium pseudotuberculosis*, has a valid claim to being the “perfect parasite”. Once successfully established within the host, this pathogen can evade the immune system with apparent ease. As a result, chronic infections tend to last for most or all of an animal's life, although they are rarely fatal (Valli 2007). If left unchecked, the disease may infect the majority of sheep in a flock. Even away from its mammalian host, the organism is well equipped for long-term survival in the environment. As a result of these formidable characteristics, flock infections by *C. pseudotuberculosis* are generally “managed” rather than eradicated.

History

In 1888, the French bacteriologist Edward Nocard isolated an unusual organism from a case of lymphangitis in a cow (Nocard 1896). Some three years later, the Bulgarian bacteriologist Hugo von Preïsz identified a similar bacterium in cultures from a renal abscess in a ewe (Preïsz and Guinard 1891). Two years later Nocard isolated the same organism from a horse thought to be suffering from glanders. As a consequence of these related discoveries, the organism in question became known as the "Preïsz-Nocard"

bacillus, a vernacular name with which it was linked for decades thereafter. Towards the end of the century caseous lymphadenitis was described for the first time in the United States (Norgaard and Mohler 1899).

Towards the end of the 19th Century the bacterium was described by the German bacteriologists Lehmann and Neumann in the first edition of their bacteriological atlas (Lehmann and Neumann 1896). In that publication, the Preisz-Nocard bacillus was renamed *Bacillus pseudotuberculosis* - a derivation of the Greek *pseudes tuberculosis* or "false tuberculosis" and a reference to the supposed clinical similarity of the lesions to caseous nodules of mycobacterial tuberculosis. In the First Edition of Bergey's *Manual of Determinative Bacteriology*, published in 1923, the organism was placed in the *Corynebacterium* genus, which had originally been created as a category for the human pathogen *Corynebacterium diphtheriae* (Benham and others 1962). This edition of the *Manual* referred to work showing that *B. pseudotuberculosis* resembled *C. diphtheriae* in morphology and cell wall composition, leading to a further change of name to *Corynebacterium ovis*. Subsequently however, the organism was isolated from purulent infections and ulcerative lymphangitis in other mammalian species, including goats, horses and human beings (Daines and Austin 1934; Merchant 1935). In recognition of this, by the time the Sixth Edition of Bergey's *Manual* was published in 1948, the species name had been changed back from *ovis* to the earlier designation of *pseudotuberculosis* (Benham and others 1962). Since that point in time, the officially recognized designation of *C. pseudotuberculosis* has remained consistent (Euzéby 2010); notwithstanding the fact that several authors continued to refer to the organism as *C. ovis* until the 1980s (Carne and Onon 1978; Burrell 1980; Nain and others 1984; Shnawa and others 1988).

Geographical Distribution

The global range of *C. pseudotuberculosis* reflects that of farmed small ruminants, the bacterium having been identified in Europe, Australasia, North and South America,

Africa and the Middle East (Robins 1991; Paton and others 2005). In many countries, CLA has for decades been an established and economically important infection of livestock, and particularly sheep (Merchant 1935). As early as the 1930s it was acknowledged that the disease “very extensively affected” the flocks of most mutton exporting countries of the southern hemisphere (Cesari 1930). It is surprising therefore, that the first diagnosed case in an animal originating from the UK was not confirmed until as recently as 1990, and even then one of those reports concerned goats (Lloyd and others 1990a), whilst the other was in sheep (Meldrum 1990).

Some suggestions have been made that the origins of the infection may lie in Europe, and that spread of *C. pseudotuberculosis* around the world followed the exportation of sheep, by the 18th Century colonial powers (Paton 2000). The Merino breed, which originated in Spain, was widely valued as a dual meat and wool animal and was exported extensively, first to South Africa and subsequently to Australia and the Americas. This early exportation may, at the very least, have assisted in the spread of *C. pseudotuberculosis* infection, a condition to which the Merino as a breed is particularly prone (Paton 2000; West and others 2002). Such a theory is difficult to prove, but supporting evidence is provided by the highly conserved nature of the *C. pseudotuberculosis* genome, irrespective of the country from which the strain originates (Connor and others 2007). Certainly, it is interesting to speculate that the absence of the Merino as a commercial breed in the British Isles may have gone some way to protecting this country from the infection.

Equine *C. pseudotuberculosis* infections became a serious problem for military planners during the First World War, when widespread infection caused the debilitation of hundreds of horses used on the battlefields of Europe (Mitchell and Walker 1944). Ovine CLA later sparked controversy in Britain during the 1920s, when mutton carcasses imported from a number of different countries were found to be affected by the disease. It was noted at the time that evidence of infection would often remain unobserved at import meat inspection "only to be revealed when the roast leg of mutton appears on the

table and is cut through" (Rolleston and Wooldridge 1926). Mutton from Argentina was particularly badly affected, forcing the British government of the time to take action in relation to consignments of carcasses from that country (Anon 1929). The extent of the problem was demonstrated by a report that in just one season, 9,770 mutton carcasses (representing 27% of the total throughput) were rejected at a single Patagonian meat plant because of CLA (Mills 1928). Other mutton exporting countries took heed of the concerns in Britain and it was soon acknowledged that "Argentina, Uruguay, Chili (sic), Australia and New Zealand are actively occupied...in a struggle against this tenacious chronic disease which, completely neglected up to now, is very extensively affecting the flocks of those countries" (Cesari 1930). These concerns also stimulated veterinary research into the disease, principally in Australia, leading to important early discoveries on pathogenesis, bacterial survival and risk factors (Seddon and others 1929; Bull and Dickinson 1935). As early as the 1920s a "preventative vaccine" was said to be used in France to protect sheep from CLA infection (Howarth and Young 1926)

Research interest in CLA was renewed in the 1970s, when the authorities in the USA, Canada and Japan applied strict regulations relating to the presence of lesions in imported sheep carcasses. A further series of studies into disease pathogenesis and epidemiology was then initiated in Australia (Nairn and Robertson 1974; Burrell 1978a; Batey 1986b, Batey 1986c), which, as a major exporter of sheep, with a high CLA prevalence within its national flock, had much to lose from the stricter regulatory regime. This in turn led to the formulation of control strategies aimed at reducing disease prevalence (Nairn and others 1977; Ellis and others 1987; Paton and others 1988a) and provided a catalyst for developments in the field of vaccination (Nairn and others 1977; Eggleton and others 1991a; Eggleton and others 1991b; Eggleton and others 1991c; Paton and others 1995).

Global Prevalence of CLA

While *C. pseudotuberculosis* is recognized as having a worldwide distribution, farm and abattoir-based research aimed at establishing disease prevalence rates, has been limited to relatively few countries. The average prevalence of CLA amongst adult sheep in Western Australia was recorded as 58% in 1973, and as 53% in 1984 (Batey 1986a). In an Australian abattoir survey, 54% of adult ewes and 3.4% of lambs showed evidence of infection at meat inspection (Batey 1986b). In another abattoir survey, individual flocks in Tasmania and Western Australia demonstrated prevalence levels within the adult population as high as 61% (Middleton and others 1991). Subsequent surveys tended to identify steadily decreasing prevalence rates, this generally being attributed to the introduction of a CLA vaccine in 1983 and its increasing acceptance within the farming community (Paton and others 2003). A field study of 412 sheep flocks, once again conducted in Western Australia, recorded an average prevalence of 45% (Pepin and others 1994a). A combined abattoir and postal survey of farmers carried out in 2002, suggested that the average prevalence had fallen to 20% in Western Australia, 23% in Victoria and 29% in New South Wales (Paton and others 2003).

A survey conducted in 1986/87 by meat inspectors in New Zealand identified lesions of CLA in 7.1% of the adult sheep slaughtered and 0.64% of lambs (Nuttall 1988). CLA was also identified as the leading cause of sheep carcass condemnation in South African abattoirs (Collett and others 1994) where losses of between 0.24 and 0.3% of all sheep carcasses were attributed to CLA and substantial additional losses were incurred due to carcass trimming (Paton and others 1995). In the western USA, average disease prevalence amongst adult ewes was estimated to be as great as 42.5% (Stoops and others 1984). Similar studies have been conducted in the Canadian province of Quebec, where the prevalence of clinical CLA was found to range from 21 to 36% amongst culled adult sheep (Arsenault and others 2003). Abattoir statistics from Alberta indicated that up to 5% of mutton carcasses and 0.03% of lamb carcasses were condemned due to CLA, and

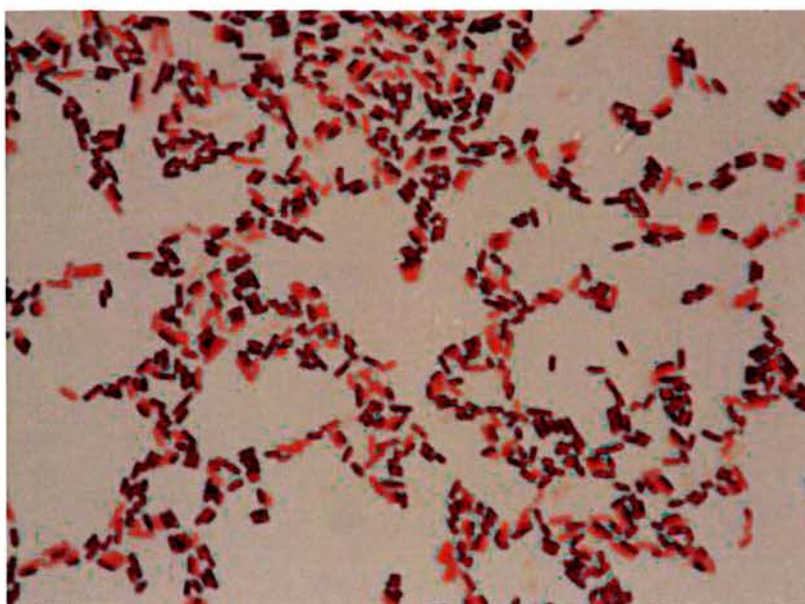
that a further 8% of all carcasses were trimmed to remove CLA lesions (Stanford and others 1998). Unfortunately, similar published statistics do not exist in respect of Europe. In the UK, where small abattoir surveys of CLA prevalence have been carried out, the results to date have been of limited scope and significance (Mechie 1998).

Bacterial Characteristics

Classification

The Corynebacteriaceae are now considered to belong to the Corynebacterineae, a suborder of Actinomycetales. The Corynebacterineae also includes most of the acid-fast bacteria, including the *Mycobacterium* and *Nocardia* genera (Clarridge and Spiegel 1995) characterised by the high mycolic acid content of the cell wall. *Corynebacterium pseudotuberculosis* possesses many of the classical features of its genus (Collins and Cummins 1986). It consists of non-motile pleomorphic rods (0.5-0.6 µm by 1.0-3.0 µm) which are Gram-positive, although staining may sometimes be irregular. Groups of the bacteria tend to show a characteristic palisade or “Chinese letter” arrangement in smears (Figure 1.1).

Figure 1.1 Gram-stained smear of *C. pseudotuberculosis* organisms showing irregular staining and formation of palisades (picture supplied by Kathleen Connor).



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

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

The so-called chemotaxonomic approach has been used widely as a means of classification of bacteria, based upon an analysis of the chemical composition of the cell wall. Analysis of cell wall mycolic acids greatly aided clarification of the taxonomy of actinomycetes, especially those of the genera *Corynebacterium*, *Mycobacterium*, *Rhodococcus* and *Nocardia* (Minnikin and others 1975; Goodfellow and others 1976; Keddie and Cure 1977; Minnikin and Goodfellow 1980; Collins and others 1982). In these studies, thin-layer (pyrolysis) chromatographic analysis of mycolic acid revealed that fatty acid chain length varied according to the genus and to a lesser extent the bacterial species. It was shown that mycobacterial mycolic acids normally consist of chain lengths of between 60 and 90 carbon atoms, and may possess a number of distinct functional groups (Minnikin and others 1978). In contrast, nocardiae and rhodococci

were shown to possess shorter mycolic acids, consisting of between 36 and 66 carbon atoms, and with fewer functional groups (Minnikin and Goodfellow 1976). The mycolic acids of corynebacteria were found to be even smaller, being between 20 and 36 carbon atoms in length, and usually saturated or containing a single double bond (Minnikin and others 1978). Collins and others (1982) reported that, consistent with the mycolic acid classification of corynebacteria, strains of *C. pseudotuberculosis* possessed mycolic acids with carbon chain lengths of between 26 and 36 carbon atoms, which contained predominantly saturated side-chains of 14 carbon atoms length.

There are few published data on the resistance of *C. pseudotuberculosis* to chemical disinfectants. However, most common disinfectants, including calcium hypochlorite, formalin and cresol solution, appear to be effective in killing the organism, but the presence of organic material necessitates increased exposure time (Ismail and Hamid 1972). This protective effect is clearly significant for a pathogen commonly found within a thick matrix of purulent debris. The organism is capable of surviving in commercial sheep dip solutions for 24 hours or more, a point of relevance in disease control (Nairn and Robertson 1974).

Early reports showed that *C. pseudotuberculosis* isolates from different mammalian species shared identical biochemical characteristics, with the exception of nitrate reduction. It was found that the majority of isolates from horses and cattle reduced nitrate to nitrite, while those from sheep and goats did not (Knight 1969). This led to the proposal of two distinct biotypes or subspecies (Biberstein and others 1971), organisms responsible for disease in sheep being consistently nitrate-negative (Sutherland and others 1996). Based on this property of nitrate reduction, Songer and others (1988) proposed the designations *C. pseudotuberculosis* biovar *ovis* (biotype 1) and *C. pseudotuberculosis* biovar *equi* (biotype 2). However, the isolation in recent years of nitrate-negative strains of *C. pseudotuberculosis* from cattle in Israel (Yeruham and others 1997) and from horses in the UK (Connor and others 2000), suggests that such a form of categorization may be unsatisfactory.

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

Virulence factors

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

Phospholipase D

The designation "phospholipase" is used to describe a varied group of enzymes able to hydrolyse one or more ester linkages in glycerophospholipids; the letters A-D are used to distinguish between phospholipases and to denote the specific phospholipid ester bond that is cleaved (Ansell and Hawthorne 1964). In eukaryotic cells, phospholipase enzymes play a role in signal transduction and normal membrane maintenance. Some mammalian cells also produce phospholipases as part of the cellular inflammatory response (Schmiel and Miller 1999). Eukaryotic cell membranes are composed of proteins and lipids, which constitute a significant target of attack during microbial invasion of host tissues. As part of their invasive arsenal, many microbes have developed their own phospholipase enzymes, which may be used to hydrolyse phosphate bonds within membrane phospholipids (Ghannoum 2000). The result of this is the damage or destruction of host cell membranes, which in turn may lead to their

dysfunction or disruption, or both (Salyers and Witt 1994). Various bacterial genera are known to secrete phospholipase enzymes, and in some cases these have been shown to play a role in virulence (Schmiel and Miller 1999). In *C. pseudotuberculosis*, PLD has been identified as a potent exotoxin and a key virulence factor in the development of CLA (Carne and Onon 1978).

PLD in this organism was first characterized by (Carne 1940) and has since been detected in every isolate of *C. pseudotuberculosis* that has been studied; including isolates of both of the suggested biotypes, and all known strains of the organism recovered from infected mammalian species (Songer and others 1988). The contention that PLD represents a significant virulence factor is supported by much experimental evidence. Isolates of *C. pseudotuberculosis* in which the *pld* gene, encoding PLD, has been deleted from the chromosome or rendered inactive by mutation, are incapable of causing the classic lymph node abscesses of CLA in sheep (Hodgson and others 1992; McNamara and others 1994). Similarly, the presence of specific antibody to PLD greatly limits the progress of the clinical disease (Brown and others 1986).

PLD is defined as a sphingomyelin-specific phospholipase that catalyses the dissociation of sphingomyelin into ceramide phosphate and choline (Bernheimer and others 1980; Pepin and others 1994a). The *C. pseudotuberculosis pld* gene has been cloned and sequenced, and analysis reveals that it encodes a protein of some 31.4 kDa, preceded by a probable secretory signal sequence of 2.7 kDa (Hodgson and others 1990). The relatively large size of the protein molecule assists in its purification in the laboratory and enables large quantities to be collected (Egen and others 1989).

Several biological activities have been reported for PLD including dermonecrosis (Carne 1940; Muckle and Gyles 1986), lethality (Brogden and Engen 1990), synergistic lysis of erythrocytes in the presence of an extracellular *Rhodococcus equi* factor (Fraser 1961), and inhibition of staphylococcal lysin-induced lysis of erythrocytes (Zaki 1976); the two latter activities are employed as laboratory tests for the identification of *C.*

pseudotuberculosis. PLD also interferes with ovine neutrophil chemotaxis and is lethal to the cells themselves (Yozwiak and Songer 1993). In terms of the significance of PLD as a virulence factor, the activity that has been the focus of most interest is the increase in vascular endothelial membrane permeability engendered by the hydrolysis of sphingomyelin. This increased permeability leads to the leakage of plasma from blood vessels and into the surrounding tissues, and from there into the lymphatic drainage (Jolly 1965; Carne and Onon 1978). This effect may assist pathogenesis by favouring the lymphatic drainage of *C. pseudotuberculosis* in tissue fluid (Batey 1986c).

PLD may assist the organism at the site of initial infection in other ways. It is known to activate the complementary pathway of the innate immune system, thereby depleting complement in the region surrounding the invading bacteria and protecting them from opsonization (Yozwiak and Songer 1993). The same paper indicates that it may also impair the chemotaxis of neutrophils and, as a consequence, decrease the likelihood of phagocytosis early in infection. In some respects this suggestion is at odds with other theories of pathogenesis, which propose that in the early stages of disease the organism parasitizes phagocytic cells and multiplies within them. Indeed, other authors have indicated that PLD may play a role in the escape of the bacterium from within macrophages (Pepin and others 1994a). It is possible that this role is related to the action of PLD on the inner phospholipids layers of the macrophage cell membrane, as indicated by the comparable observations on other bacterial infections (Titball 1993).

Exotoxins with similarities to the PLD of *C. pseudotuberculosis* are known to be important virulence factors for other bacterial pathogens. For example, there is 97% homology between *C. pseudotuberculosis* PLD and the active exotoxin of *C. ulcerans*, a rare cause of human diphtheria (McNamara and others 1995). A PLD-like exotoxin is also considered to be an important virulence factor in *Pseudomonas aeruginosa* (Wilderman and others 2001). Likewise, the gene coding for *C. pseudotuberculosis* PLD (*pld*) shows homology to the *ymt* gene from the plague-causing organism *Yersinia pestis* (Hinnesbusch 2000). PLD also demonstrates an intriguing similarity in structure

and biological activity to an enzyme toxin produced by the venomous North American brown recluse spider, *Loxosceles reclusa* (Bernheimer and others 1985). This toxin induces dermonecrosis, haemolysis, platelet-aggregation and, on rare occasions, fatal renal failure (Lee and Lynch 2005). Similarly, it has been shown that severe haemolytic crises may result from the injection of *C. pseudotuberculosis* culture supernates or crudely purified exotoxin preparations into small ruminants (Hsu and others 1985).

Mycolic acid

C. pseudotuberculosis does not produce a protective capsule but has instead a waxy mycolic acid coat on the cell wall surface. Observations on the growth of virulent and attenuated strains of *C. pseudotuberculosis*, together with quantitative differences in the amount of extractable mycolic acid from two of these strains, led to the hypothesis that these surface lipids were concerned with pathogenicity and virulence (Jolly 1966).

Later work established the cytotoxic properties of this lipid coat, which was shown to play a major role in pathogenicity (Hard 1972; Muckle and Gyles 1983; Tashjian and Campbell 1983). The subcutaneous injection into mice of mycolic acid extracted from *C. pseudotuberculosis* results in localized swelling, with congestion and a central area of haemorrhagic necrosis (Muckle and Gyles 1983). Similarly, when guinea pigs are injected the inoculated area of skin becomes blanched, although surrounded by a zone of erythema. This is then followed by local necrosis and tissue sloughing (Hard 1975). In addition, mycolic acid induces degenerative changes and death in phagocytising leucocytes (Carne and others 1956). However, unlike the lethal effect of injection of similar molecules extracted from mycobacteria, the cytotoxic effect of *C. pseudotuberculosis* mycolic acid is confined to the site of injection (Carne and others 1956; Hard 1975).

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

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

Other secondary factors involved in infection

Undetermined differences in virulence factors have been suggested to account for the specific “ovine/caprine” and “equine/bovine” strains of *C. pseudotuberculosis*, which are in other respects (cultural, antigenic and toxigenic) equivalent (Valli 2007). The fact that the equine disease syndromes associated with the organism appear to be absent from large parts of the world in which the ovine infection is endemic, supports the existence of such differences. As discussed later, the probable importance of secondary factors,

such as insect vectors in *C. pseudotuberculosis* infections of horses (Miers and Ley 1980; Spier and others 2004), may go some way to explaining this. However, in order to clarify the matter fully, experimental infections with ovine and equine strains in parallel would be required. A previously unreported 40 kDa protein antigen of *C. pseudotuberculosis*, suggested to be a virulence factor (Wilson and others 1995) has yet to receive detailed study.

Clinical and Pathological Features of Caseous Lymphadenitis in Sheep

C. pseudotuberculosis infections in sheep are classically associated with the formation of pyogranulomas (Valli 2007), and this accounts for the name “caseous lymphadenitis”. The lesions occur in two main forms, namely external (also known as superficial or cutaneous) and internal (or visceral), which may co-exist within the same animal.

The external form is characterized by abscessation of those lymph nodes that may be seen or palpated externally (Figure 2.2). Any of the superficial lymph nodes of the body may be affected, dependent upon the original point of entry of the organism. Less commonly, localized purulent lesions not directly associated with the superficial lymph nodes may occur within the subcutaneous tissues. Such lesions may appear as organized abscesses, with swelling, fibrous encapsulation, loss of overlying hair and eventual rupture, resulting in the discharge of pus (Radostits and others 2000a).

The visceral form is associated with abscesses in the internal lymph nodes and other organs. In sheep, the principal locations for these internal CLA lesions are the lung parenchyma and mediastinal lymph nodes. Lesions may also be found in the liver, kidneys or udder, and more rarely the heart, testis, scrotum, uterus, joints, brain or spinal cord (Valli 2007).

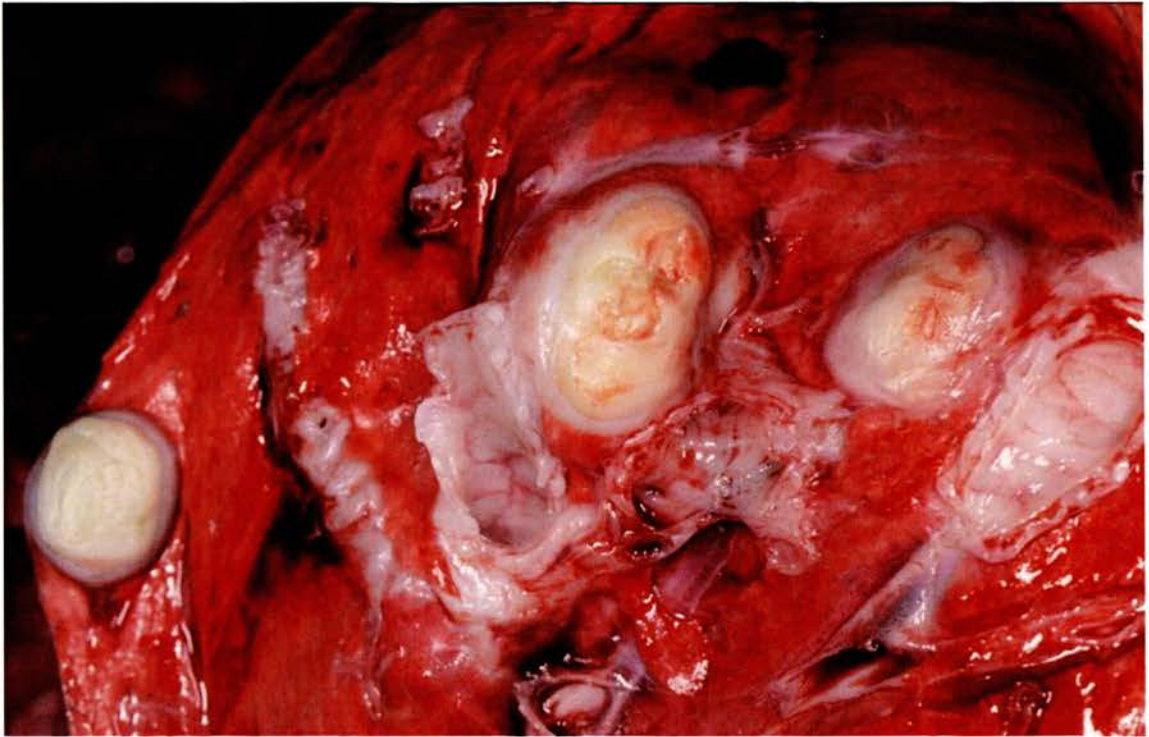
Figure 2.2 A typical external CLA lesion in the parotid lymph node of an adult Friesland ewe which is yet to discharge its purulent contents (picture taken by author).



Pathogenesis in sheep

There is a general consensus among recent reviewers regarding the stages through which *C. pseudotuberculosis* infection progresses (Batey 1986c; Pepin and others 1994a; Paton and others 2005). After initial entry, the organism spreads rapidly to the local drainage lymph node. Here, multiple microscopic pyogranulomas develop, growing in size and coalescing to form larger abscesses. This is sometimes followed by a further extension of infection via the blood or the lymphatic system, leading to similar lesions in other organs. The nature of these slowly developing CLA lesions means that chronic, and frequently lifelong, disease is the rule rather than the exception (Valli 2007). Viable bacteria may be recovered from abscesses several years after initial infection (Williamson 2001). Reactivation of disease may also occur, with the growth of lesions recommencing after a period of apparent quiescence (Brown and Olander 1987; Pepin and others 1994a).

Figure 2.3 CLA abscesses within the lung parenchyma. Note the close proximity of the lesions to the small airways (picture taken by author).



Numerous routes of inoculation have been used to induce experimental CLA in sheep; intradermal, subcutaneous, intravenous, intratracheal, intravaginal, and intralymphatic inoculation have all proved successful in establishing disease (Nagy 1976; Burrell 1978a; Pepin and others 1994b; Fontaine and others 2006). In natural infections however, the principal route of entry is believed to be through the skin (Batey 1986c; Brown and Olander 1987; Davis 1990; Collett and others 1994). This initial infection is facilitated by minor cutaneous wounds and abrasions, especially those caused by shearing (Paton and others 1988a). Wounds caused during castration or docking have also been suggested as an occasional route of entry, as has the umbilicus in neonatal animals (Valli 2007). Entry via the oral cavity has been postulated to account for the small number of head and neck lesions seen in sheep from the Antipodes and North America, and similar, more common lesions in goats (Ashfaq and Campbell 1979). In

contrast, the more distal parts of the intestinal tract are not believed to provide a portal of entry for the organism, even following parasitic damage (Valli 2007).

A respiratory route of CLA infection has been postulated (Stoops and others 1984) and has been widely quoted in subsequent reviews (Batey 1986c; Brown and Olander 1987). This theory was based on the observations that some naturally infected sheep show only pulmonary lesions, and that a small number of these lesions are located within the walls of airways (Figure 2.3). Moreover, the production of disseminated pulmonary abscesses has been reported to occur following the intratracheal injection of a broth culture of *C. pseudotuberculosis* (Brown and Olander 1987). However, other studies have indicated that such pulmonary lesions may develop as part of a systemic infection initiated elsewhere in the body. Thus, after the intravenous inoculation of lambs with *C. pseudotuberculosis*, the majority of internal lesions appeared in the lungs and associated thoracic lymph nodes (Brogden and others 1984b). It has also been noted that in natural ovine CLA infections, the patterns of distribution of pulmonary lesions are consistent with haematogenous or lymphogenous spread rather than with aerogenous spread (Nairn and Robertson 1974). It would therefore appear that entry of infection via the respiratory tract, although a theoretical risk, is of minor importance.

As already described, purulent cutaneous or subcutaneous lesions from which *C. pseudotuberculosis* may be isolated, occur infrequently in sheep, even in flocks in which the incidence of classic lymph node lesions is high. It therefore follows that, on infecting the host via the skin, the organism is poorly suited to establishing persistent infections at the site of entry and on most occasions must progress to the local drainage lymph node and beyond if infection is to be sustained (Valli 2007). The means by which the organism spreads from the initial point of entry, eventually to form the classic lesion of CLA, has received close examination (Brogden and others 1984b; Holstad and Teige 1988; Pepin and others 1991a; Pepin and others 1991b). Of crucial importance is the survival and replication of *C. pseudotuberculosis* in macrophages, which then carry the organism to the site of the eventual CLA lesion.

The use of radioisotopically labelled inflammatory cells and scintigraphic imagery demonstrated that within a few hours of subcutaneous inoculation with bacteria, huge numbers of neutrophils were recruited to the injection site and by 24 hours post-inoculation, these neutrophils began to appear in the local drainage lymph node (Guilloteau and others 1990). The relative number of neutrophils decreased from day three, while the relative numbers of macrophages at the site of inoculation rose dramatically. As suggested above, the ability of *C. pseudotuberculosis* to survive phagocytosis by such cells and to exist as a facultatively intracellular parasite, enables the organism to be carried within these cells via the lymphatic drainage to the local lymph node (Pepin and others 1994a). Thus, it is thought that phagocytic cells recruited to the area in response to infection become the very means by which further colonization of the body is achieved.

Once a lymph node has been colonized by *C. pseudotuberculosis* it undergoes a short period of generalized inflammation. PLD produced by the *C. pseudotuberculosis* organisms is the probable initiator of this lymphadenitis. It has been reported that within 24 hours of the subcutaneous inoculation of lambs a number of micro-abscesses occurred within the cortical region of the lymph node draining the site of inoculation (Pepin and others 1991b). By day six post-inoculation, these micro-abscesses had become more numerous and began to expand and coalesce to form larger purulent foci. The early pyogranulomas contained clumps of bacteria and cellular debris, with a relatively high proportion of eosinophils giving the purulent core a slightly green hue macroscopically. At the same time, and in parallel with the cellular events at the point of entry, the infiltration of neutrophils diminished and monocytes/macrophages became the predominant cell type within the lesion (Pepin and others 1994b). A process during which the lesion became encapsulated followed shortly thereafter, leading to a diminution of the inflammatory reaction in the parenchyma of the node. Continued slow expansion of the lesion sometimes then occurred, depending on the location of the node and whether or not it ruptured to discharge its contents. Such enlargement progressed

through a repeated cycle of necrosis and reformation of the outer capsule. In the early stages, the purulent contents of the abscess were soft and semi-fluid; however, as time progressed, the pus within the lesion took on a more plastic or solid form, in which scattered clumps of bacteria were sometimes noted. Small nodules of mineralization formed within the purulent material, causing it to become progressively paler in colour. These calcified foci tended to be laid down in concentric layers reminiscent of the cross-sectional view of an onion.

The classic "onion ring" presentation (Figure 2.4) is regarded as virtually pathognomonic for CLA in countries such as Australia and New Zealand, but is rarely reported in the UK. This probably reflects the relatively small number of chronic infections amongst sheep in Britain, where *C. pseudotuberculosis* is still regarded as a new and emerging disease and infected animals are still likely to be culled after diagnosis (Baird 2003). Superficial lymph node abscesses may expand to reach a diameter of as much as 15 cm, but 3-5 cm is a more common size (Valli 2007).

The majority of reviews of ovine CLA have been written from a North American or Australian perspective. These describe the external form of CLA as most commonly affecting the superficial lymph nodes of the torso (Nagy 1971; Stoops and others 1984; Brown and Olander 1987; Williamson 2001). A review of the disease in US sheep, reported that the precrucial node was most commonly affected, followed by the prescapular and then by other superficial lymph nodes with equal frequency (Ayers 1977). The author regarded the occurrence of lesions in the head and neck as relatively rare in sheep, but more common in goats. A similar statement is made by an Australian author describing the usual presentation of the disease in that country (Batey 1986b). Authors from New Zealand (Nuttall 1988), South Africa (Collett and others 1994) and Canada (Muckle and Menzies 1993) all describe a similar tendency for superficial lesions to develop particularly in the lymph nodes of the torso. This therefore implies, for the above countries, that the skin of the torso is a frequent site of entry for the pathogen.

Figure 2.4 Superficial CLA lymph node abscess in an aged ewe in Perth, WA. Note the typical onion ring appearance as recognised commonly in Australia (picture taken by author).

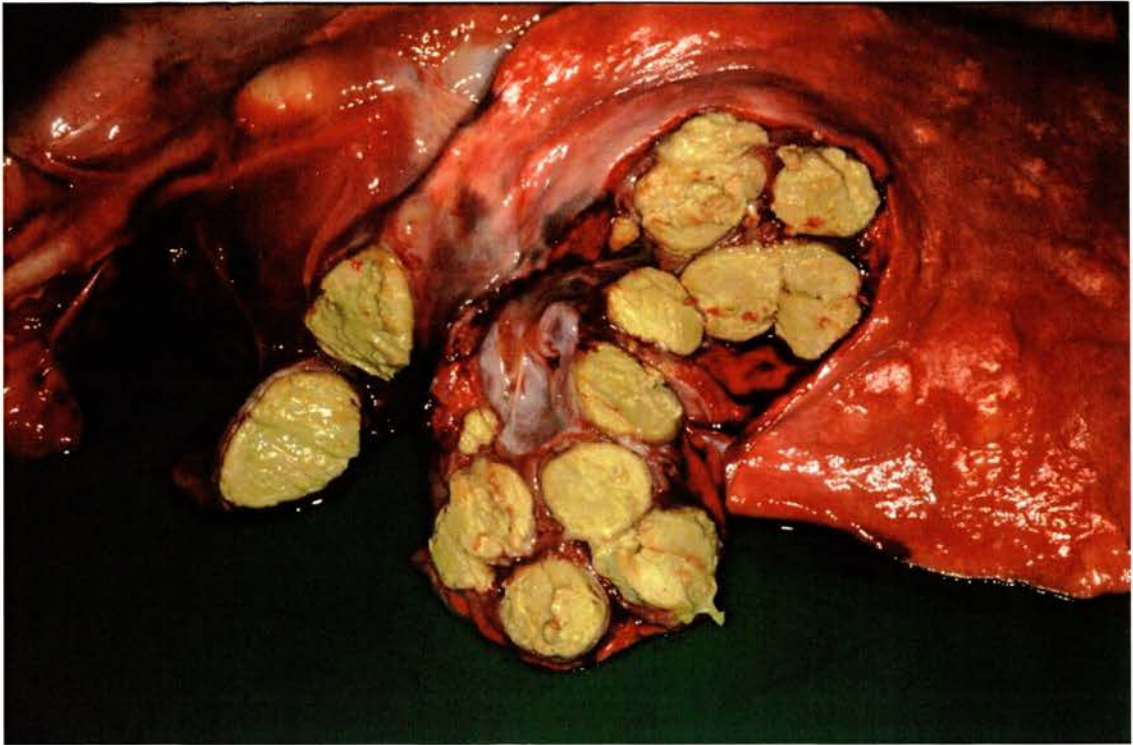


It is likely that the infected superficial lymph node represents a staging post for the further colonization of internal lymph nodes and viscera. At which stage in the course of the infection such colonization occurs, is the subject of some speculation. It seems probable that purulent emboli detach from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

Other than the lymph nodes, the lung is the most frequent site of CLA lesions (Stoops and others 1984; Brown and Olander 1987; Pekelder 2000; Paton and others 2005). As already noted, the generally random distribution of lesions within the parenchyma is consistent with haematogenous or lymphogenous spread, with seeded lesions developing from within the alveolar vasculature (Batey 1986c). These pulmonary lesions most commonly take the form of encapsulated abscesses similar to those seen in the lymph nodes (Figure 2.5), although occasionally a more extensive bronchopneumonia is also recorded (Valli 2007). The latter may lead to areas of pleuritis, resulting in the development of fibrinous or fibrous adhesions to the chest wall, pericardium or diaphragm (Renshaw and others 1979; Stoops and others 1984). In animals with pulmonary abscesses, CLA lesions are commonly encountered in the associated mediastinal and bronchial lymph nodes, implying an additional step in the transit of the organism from the lung parenchyma. Abscesses within the mediastinal lymph nodes may become so large as to put pressure on the adjacent oesophagus, interfering with normal swallowing and rumination, and leading to chronic ill-thrift (Valli 2007).

Mastitis due to *C. pseudotuberculosis* is encountered occasionally in sheep and is most likely to represent an extension of infection from the adjacent supra-mammary lymph node (Radostits and others 2000a). It may take the form of an acute suppurative mastitis, or appear as chronic encapsulated abscesses within the mammary gland (see Figure 4.1). Lesions in other organs such as the liver, kidneys and scrotum also tend to be encapsulated abscesses containing a thick caseous material, which may again take on a lamellated form in chronic cases (Valli 2007). On rare occasions, the organism has also been isolated from the stomach contents and tissues of ovine foetuses, consistent with a role in abortion (Dennis and Bamford 1966).

Figure 2.5 Multiple encapsulated CLA abscesses within the lung of an adult ram examined at SAC Centre St Boswells (picture taken by author).



Economic significance of the disease in sheep

CLA is recognized as a significant cause of financial loss to the sheep industry in a number of countries where the disease is endemic. The major cause of these economic losses lies in the condemnation and downgrading of affected carcasses at slaughter and meat-inspection. In Australia, where CLA-related losses have been extensively studied, it was estimated that in the early 2000s the disease costs the meat industry \$A12 to \$A15 million per annum (Paton and others 2003). This was due both to carcass losses and to the requirement for additional staff in abattoirs to carry out meat inspection and carcass trimming.

Systemic infection by *C. pseudotuberculosis* is acknowledged to be detrimental to the productivity of the infected animal, but to what extent is unclear. Studies of Merino sheep in Australia failed to show a relation between carcass conformation and weight,

and the occurrence or extent of CLA lesions (Batey 1986b). However, workers in that country established that CLA infection had a detrimental effect on wool production. In a comparison between CLA-affected and unaffected Merino sheep in three Australian flocks, the loss of total clean wool production in the infected animals was assessed as 4.1 to 6.6% during the year of initial infection (Paton and others 1988b). Based on disease surveillance and wool production data from 1992, it was estimated that CLA infection cost the Australian sheep industry approximately \$A17 million per annum in lost wool production (Paton and others 1994). However, the same field workers were unable to confirm that CLA infection had any detrimental effects on the live weight gain of young sheep (Paton and others 1988b).

In contrast to much of the Southern Hemisphere, where the systemic effects of CLA on the individual sheep are regarded as marginal, in North America the disease is considered to be much more significant clinically. There, the visceral form of infection with *C. pseudotuberculosis* has been associated with so-called "thin-ewe syndrome", a chronic emaciation of ewes, occurring despite good appetite and in the absence of significant parasitic infestation or specific clinical signs (Paton and others 2005). Two studies conducted in the US concluded that CLA infection had an economically significant effect on culling rates and reproductive efficiency in ewes (Gates and others 1977; Renshaw and others 1979), although these studies have been criticised for a lack of experimental controls (Arsenault and others 2003). Certainly, the diagnostic criteria of "thin-ewe syndrome" are vague, and although *C. pseudotuberculosis* is the principal bacterial causative agent, other organisms such as species of *Moraxella*, *Arcanobacterium* and *Staphylococcus* may also be isolated from lung lesions in mixed culture (Renshaw and others 1979). On occasion, *Moraxella* species may be present in the absence of *C. pseudotuberculosis*. More significantly, there would appear to be some evidence of a statistically significant association between the presence of CLA thoracic lesions and maedi visna-induced pneumonia in culled sheep in Canada (Arsenault and others 2003).

In the Middle East, considerable economic losses arise through the condemnation of lamb carcasses with CLA lesions, most commonly in the submandibular lymph nodes. Prevalence rates of 20-40% have been reported amongst lambs from intensively reared flocks of Najdi sheep (Pepin and others 1994a). CLA lesions in lambs destined for slaughter as part of religious festivals may render the carcass virtually worthless – equivalent to a loss of \$200 per head.

Epidemiology of Infection in Sheep

Much CLA research has focussed on how *C. pseudotuberculosis* cells, commonly contained within thick-walled abscesses, are transferred from one animal to another. Several possible routes have been suggested. The numbers of viable bacteria in purulent discharge from a CLA lesion have been estimated as between 10^6 and 5×10^7 /g (Brown and Olander 1987). The rupture of superficial abscesses therefore releases huge numbers of viable bacteria on to the adjacent skin and fleece, and may readily contaminate the immediate environment. Other animals may then be exposed, either by direct physical contact with the affected animal, or indirectly via contaminated fomites. In addition, given the ability of *C. pseudotuberculosis* to survive within the environment for a number of weeks or months, the potential period for infection may extend well beyond the point of initial rupture and discharge.

The recovery of organisms from the faeces of infected animals was reported from the earliest days of CLA research (Seddon and others 1929) and its potential significance for disease transmission has been noted in subsequent reviews (Henton 2001; Paton and others 2005). However, in the absence of CLA lesions in the intestinal tract, the organisms probably originate from the respiratory tract, or represent bacteria that have traversed the gut, having been ingested. The fact that bacteria have also been isolated from the faeces of uninfected sheep would tend to support the latter hypothesis (Benham and others 1962). Despite the ability of *C. pseudotuberculosis* to survive in the environment for protracted periods of time, attempts to isolate the organism from the

soil in areas of endemic infection have been unsuccessful (Knight 1969). Therefore the presence of *C. pseudotuberculosis* organisms in faeces, and the consequent risk to other animals are likely to be of minor importance.

In CLA-infected sheep flocks, animals with lung lesions are thought to represent the principal source of infection for other animals (Pepin and others 1994a; Williamson 2001). These conclusions are based principally on epidemiological observations recorded in Australia, in which the seroprevalence of CLA increased rapidly in groups of sheep, despite the absence of superficial CLA lesions (Ellis and others 1987; Paton and others 1988a). In addition, *C. pseudotuberculosis* has been isolated from the tracheae of sheep with pulmonary abscesses, confirming that discharge from lung lesions into the airways does indeed occur (Stoops and others 1984; Pepin and others 1994a). The assumption is made that even a relatively small number of animals with lung abscesses can create an aerosol of infectious organisms. Under conditions of close contact and reduced airflow, as in a covered shed for example, such an aerosol might be capable of infecting a large number of animals in a short period of time (Paton and others 1996).

The usual means of entry of a new infection into a naïve flock is via the introduction of a clinically or sub-clinically infected carrier animal (Ayers 1977). A role for transmission of the bacterium by farm workers and their equipment between animals within the same flock has been established (Nagy 1976) and it has also been suggested that human activity may, on rare occasions, be responsible for introducing infection to previously disease-free flocks. In flocks with a high prevalence of CLA, shearers and their equipment are inevitably exposed to the purulent discharges of superficial CLA lesions. If subsequent decontamination measures are inadequate, it is possible that shearing gear, clothing and mobile handling equipment may act as a mechanical vector of infection to other flocks. A circumstantial link between new disease outbreaks in previously unaffected closed sheep flocks, and visits by contract shearers has been suggested in

Western Australia (Paton 2000) and Canada (P. I. Menzies, personal communication); such reports, however, remain anecdotal.

Other mechanical vectors of infection have been suggested. The investigation of an outbreak of CLA in a closed and accredited disease-free goat herd in The Netherlands identified the most likely source of infection as bales of hay purchased from another farm. This second farm was known to have a herd of goats in which CLA was highly prevalent, and infected goats had been housed in the hay shed (Dercksen and others 1996).

A number of risk factors have been suggested for the spread of *C. pseudotuberculosis* infection amongst sheep. These include the breed (West and others 2002), increasing age of animals (Pepin and others 1994a; Paton and others 1995) and a dusty farm environment (West and others 2002). However, the principal risk factor identified in Australia and other parts of the world is the minor skin damage caused during shearing of the fleece (Nagy 1976; Paton and others 1988a; Serikawa and others 1993; Paton and others 1996; Al Rawashdeh and Al Qudah 2000). In Australia, sheep are frequently housed or yarded together for a period immediately after shearing, before being returned to their grazings. This spell of close confinement is regarded as the period of greatest risk, during which the organism may pass via an infectious aerosol from one animal to another (Paton and others 1996). The risks inherent in shearing may be increased if a plunge or shower dipping is applied within a few days of shearing (Nairn and Robertson 1974; Augustine and Renshaw 1986; Ayers 1986; Paton and others 1996), the suggestion here being that organisms from affected animals persist in the dip solution for a period of time.

Comparison with infection in goats

Natural CLA infections in goats demonstrate a number of similarities with the disease in sheep, not least that the same biotype of *C. pseudotuberculosis* is responsible. However, consideration of the differences in clinical presentation between sheep and goats may be

instructive in respect of pathogenesis and specific risk factors. This is of particular interest in the UK, where the clinical presentation of ovine CLA is closely similar to that of the caprine infection in other parts of the world (Baird 2003).

In goats the major sites of infection are the superficial lymph nodes, visceral lesions being encountered in only a minority of animals. In sheep, however, visceral lesions, and especially lung lesions, occur more frequently and in greater numbers than in goats (Renshaw and others 1979; Hein and Cargill 1981; Ayers 1986; Muckle and Menzies 1993). In caprine CLA, the principal sites of superficial lesions are the nodes of the head and neck (Ayers 1977; Burrell 1981; Batey 1986c; Batey and others 1986; Brown and Olander 1987; Williamson 2001). As already noted, most reports of ovine CLA from other countries indicate that head and neck lesions are uncommon, the more frequent site of abscessation being the superficial lymph nodes of the torso. These observations are consistent with the notion that the main routes of infection in goats are via the oral cavity or via skin of the face and head. If bacteria are present on the skin or in the immediate environment, certain behavioural traits shown by goats, such as mutual grooming, head butting, and an inquisitive attitude (including browsing and mouthing behaviour), would increase the risk of exposure to infection. In contrast, such behavioural traits are less frequently observed in sheep. In addition most goats are neither shorn nor subjected to plunge or shower dipping.

***Corynebacterium pseudotuberculosis* infections in other animal species**

C. pseudotuberculosis is pathogenic for a variety of mammalian species. Although a wide range of warm-blooded creatures are susceptible to the infection, specific disease syndromes are recognized in a smaller subset. In addition to the globally important diseases of sheep and goats, significant syndromes are recognized in horses and dairy cattle.

Equine disease

In horses, *C. pseudotuberculosis* is the cause of three recognized disease syndromes: ulcerative lymphangitis, contagious folliculitis and furunculosis, and deep-seated subcutaneous abscessation (Miers and Ley 1980). In the US, *C. pseudotuberculosis* has also been recorded as a rare cause of abortion (Poonacha and Donahue 1995) and mastitis in mares (Addo and others 1974), and with generalized visceral abscessation (Paton and others 2005). The three main equine syndromes tend to have a distinct geographical distribution, suggesting the importance of secondary disease factors. Indeed, the western areas of Canada and the US represent the only regions in which all three syndromes have been recorded (Aleman and others 1996). In a study employing a real-time PCR assay for the PLD-encoding gene of *C. pseudotuberculosis*, evidence of bacterial carriage was detected in a significant proportion of flies collected around horses with infections. In total, three fly species were identified as potential vectors for transmission of disease with *C. pseudotuberculosis* identified in up to 20% of the houseflies (*Musca domestica*) examined (Spier and others 2004).

Ulcerative lymphangitis first gained prominence during World War I when outbreaks occurred amongst the large numbers of horses used by allied forces in continental Europe (Mitchell and Walker 1944). In clinical cases, infection appears to enter via wounds, generally those of the lower limbs. This is followed by extension to the afferent lymphatic vessels and the formation of abscesses along their course. These abscesses appear as subcutaneous nodular lesions, which rupture to discharge pus and form necrotic ulcers (Radostits and others 2000c). Such infections may persist for many months, as successive crops of ulcers develop and then resolve. Transmission commonly occurs by direct contact between horses, although an arthropod vector has also been suggested to be of importance in Africa (Addo 1983).

Equine folliculitis and furunculosis (also referred to as equine contagious acne, Canadian horsepox and equine contagious pustular dermatitis) is a much less clinically important form of disease, which probably represents a secondary infection by *C.*

pseudotuberculosis of a pre-existing seborrhoea or dermatitis (Radostits and others 2000b). The lesions tend to develop at points of contact with harnesses and other tack, and spread may occur through the use of contaminated grooming equipment. Again, the characteristic lesion is a small nodule that develops into a pustule, before rupturing to discharge purulent material. These lesions usually resolve spontaneously but may occasionally result in an ulcer at the site of infection.

Deep-seated *C. pseudotuberculosis* abscessation (pigeon fever, Wyoming strangles, false strangles) is the most serious manifestation of the infection in horses. It is usually seen as abscesses in the musculature of the chest and shoulder, and is of economic significance in much of the western US (Hughes and Biberstein 1959; Miers and Ley 1980), the incidence being greatest in the months of September to November (Aleman and others 1996; Doherr and others 1998). This probably reflects the seasonal incidence of various species of biting arthropod that act as mechanical vectors (Doherr and others 1999).

The recognized equine syndromes have not been described in the UK in recent years, despite the isolation of *C. pseudotuberculosis* from horses on a number of occasions (Connor and others 2000). The apparent significance of seasonality, environmental conditions and arthropod vectors in *C. pseudotuberculosis* infections of horses has been emphasized by a number of authors (Hughes and Biberstein 1959; Miers and Ley 1980; Welsh 1990). It would therefore appear that, although equine pathogenic strains exist in the UK, essential contributory risk factors do not. It is noteworthy that ulcerative lymphangitis was not uncommon in the UK during World War I, but this probably resulted from the introduction of infected horses from Russia and the Balkans where the condition was present (Petrie and McClean 1934; Benham and others 1962).

Bovine disease

In recent years infections of dairy cattle have been recorded in Israel, commonly in the form of deep subcutaneous abscesses that develop into granulating and discharging ulcers that respond poorly to systemic antibiotic treatment. The disease is sporadic but may sometimes occur in an epidemic form (Yeruham and others 2003a). Morbidity rates of up to 35% have been recorded in some Israeli dairy herds (Yeruham and others 1997). A less common mastitic form of disease is also recognized. This may be mild, the only sign being the appearance of clots in the milk, or severe with a greatly enlarged and tender mammary gland and complete cessation of milk production. The severe form often necessitates premature culling, as the response to antibiotic therapy is poor (Shpigel and others 1993). In a rare visceral form of disease, widespread abscessation occurs in lymph nodes associated with the upper and lower respiratory tract (Yeruham and others 2003a) and in severe cases this may lead to swelling and obstruction of airways. Finally, the organism has also been associated with a necrotic and ulcerative dermatitis of the heel in heifers, a morbidity rate of 91% having been reported in one outbreak (Yeruham and others 2003b). *C. pseudotuberculosis* has been recorded as a cause of an ulcerative lymphangitis and mastitis of cattle in countries other than Israel, albeit rarely (Purchase 1944; Adekeye and others 1980; Kariuki and Poulton 1982; Anderson and others 1990).

C. pseudotuberculosis was isolated from houseflies collected over an ulcerative lymphangitis lesion in a cow (Yeruham and others 1996). A significant proportion of flies that have fed on superficial *C. pseudotuberculosis* lesions in cattle, or on contaminated milk from animals with *C. pseudotuberculosis* mastitis have been found to harbour the organism either on their body surfaces or in their intestines, the bacterium being present in the faeces (Braverman and others 1999). It was concluded that this species of fly had a predilection for feeding on milk residues of cow teats, and therefore played an important role in harbouring and disseminating *C. pseudotuberculosis* infections in dairy herds in Israel.

Disease in other animal species

C. pseudotuberculosis is commonly identified as the cause of a purulent lymphadenopathy affecting large numbers of farmed llamas and alpacas in Chile and other South American countries (Braga and others 2006). It is also encountered sporadically as the cause of abscesses in companion camelids in North America (Anderson and others 2004). In parts of the Middle East *C. pseudotuberculosis* has been isolated from lymph node abscesses of buffaloes (Ali and Zaitoun 1999) and from similar lesions in camels (Esterabadi and others 1975; Nashed and Mahmoud 1987; Abubakr and others 1999). Sporadic natural infections have been recorded in deer (Hammersland and Joneschild 1937; Eghetti and Mc Kenney 1941), pigs (Zhao and others 1993), coypus, primates and ducks (Benham and others 1962) and a hedgehog (McAllister and Keahey 1971). Experimental infections are readily induced in laboratory species such as mice, rabbits and guinea-pigs (Carne 1940; Jolly 1965).

Zoonotic Infections

Reports of human infection with *C. pseudotuberculosis* are relatively small in number, the first published case appearing some 30 years ago (Lopez and others 1966). The majority of human cases were classified as occupational infections, affecting workers who had regular contact with sheep, such as shepherds, shearers, abattoir workers and butchers (Henderson 1979; Richards and Hurse 1985; House and others 1986; Peel and others 1997). Human infections tend to be chronic, presenting as a localized suppurative granulomatous lymphadenitis, affecting the axillary, inguinal or cervical lymph nodes (Mills and others 1997). The lymphadenitis may follow a period of influenza-like symptoms and increasing lethargy. Treatment with systemic antibiotics is generally unrewarding, the majority of cases requiring surgical excision of the affected lymph node. Even then, the healing of surgical wounds and infected sinuses may be protracted, and recurrence of the infection at the same or adjacent site is not uncommon (Peel and others 1997). Because affected human lymph nodes are not always cultured after excision and because unspecified axillary lymphadenitis is not uncommon in shearers, it has been speculated that human *C. pseudotuberculosis* infections are under-reported in

countries such as Australia, where ovine CLA is especially prevalent (Hamilton and others 1968).

More serious infections in man, which extend beyond a localised lymphadenopathy, have been recorded. On one occasion an eosinophilic pneumonia due to *C. pseudotuberculosis* was diagnosed in a veterinary surgeon from the US (Keslin and others 1979). There are however no published records of fatal infections in human patients. One case of particular note was recorded in a city-dweller, who had no apparent connection with a farming environment or livestock. In this instance, the only potential cause identified was the regular consumption by the patient of unpasteurized goat's milk (Goldberger and others 1981). No case of human infection with *C. pseudotuberculosis* has yet been reported in the UK. There is however, a reference to an isolate of *C. pseudotuberculosis* from a human lymph gland submitted to the UK National Collection of Type Cultures (Hill and others 1978), although the origin of the sample was not mentioned.

A recent report described a case of *C. pseudotuberculosis* necrotizing lymphadenitis in a 12-year-old girl in France (Join-Lambert and others 2006), thought to be the first reported childhood case. The girl contracted the infection after having had contact with sheep while on vacation in a rural part of France. The nitrate-negative organism was susceptible *in vitro* to a range of common antibiotics, but treatment with amoxicillin and clavulanic acid failed to clear the infection. A course of amoxicillin/clavulanate was then administered again without success. After the infection had increased in severity and spread beyond the affected inguinal lymph node, intravenous antimicrobial therapy with imipenem/cilastatin, rifampicin and ofloxacin was administered, followed by surgical resection of affected tissue other than lymphatic tissue (to avoid post-lymphadenectomy oedema). Intravenous antibiotic administration was continued for a further four months, followed by oral rifampicin and ofloxacin treatment for a further six months. Two years after the cessation of treatment, no relapse had occurred.

Chapter 3: SEROLOGICALLY BASED PREVALENCE STUDY OF CASEOUS LYMPHADENITIS IN RAMS FROM PEDIGREE TERMINAL SIRE BREEDS IN GREAT BRITAIN.

Summary

In order to assess the likely flock prevalence of caseous lymphadenitis infection within the GB terminal sire sector, blood samples submitted from rams as part of the Maedi Visna Accreditation Scheme were tested using a CLA ELISA assay. The assay employed detected antibody to PLD and has a reported sensitivity in sheep of $79\% \pm 5\%$ and a specificity of $99\% \pm 1\%$ when published cut-offs are applied. When blood samples produced ELISA titres in the inconclusive range these were further tested using a Western blot technique. A total of 2,538 serum samples from 745 different flocks were tested in this way. A total of 250 serum samples were considered positive for PLD antibody. This gave an overall apparent prevalence of 9.93% (with 95% confidence interval of 8.76% - 11.1%; 99% confidence interval of 8.39% - 11.46%). One or more seropositive animals were detected in 134 different flocks, representing almost 18% of the total flocks sampled. These flocks were present throughout the country and animals from all major terminal sire breeds were found to be seropositive.

Introduction

Although it is generally accepted that CLA is a relatively new arrival to the UK, there exists a tantalising report concerning the isolation of an “unusual diphtheroid” bacterium from three Swaledale sheep from the same flock in southwest Cumberland (Heath and Batty 1952). Based on colony and cell morphology and biochemical tests, these isolates corresponded almost exactly to the previously described characteristics of *C. pseudotuberculosis* (at that time *C. ovis*) reported a few years earlier in the US (Morse 1949). Realizing the potential implications, Heath and Batty arranged for further testing by other researchers in the field. The authors reported that a Dr. Lovell had

shown that although a member of the genus *Corynebacterium*, the organism was not pathogenic to guinea-pigs, did not “attack” urea and probably contained metachromatic granules; hence it was considered not to be *C. pseudotuberculosis*. In addition, a Mr. Hudson observed that the organism was not now virulent for sheep, but did otherwise resemble *C. pseudotuberculosis*. In concluding their report, the authors stated that the organism may be a different variety of the same species (i.e. *C. pseudotuberculosis*) and that it was worthy of record given the extremely rare isolation of corynebacteria (other than *C. pyogenes*) from British sheep (Heath and Batty 1952).

Given the inconclusive nature of the above report, and the absence of any further literature pertaining to *C. pseudotuberculosis* infections in the UK, the currently accepted first cases of infection in live animals in this country were confirmed in January 1990 by workers at Cambridge University (Lloyd and others 1990a). The diagnosis was made in two dairy goats from a farm in the south of England, these animals being part of a group of 20 Boer goats originally imported from the then West Germany in 1987 (Robins 1991).

C. pseudotuberculosis infection, although not a notifiable disease at that time, was still of interest to the UK State Veterinary Service. The farm on which the affected goats were kept was therefore placed under immediate movement restrictions. A subsequent tracing exercise led to the confirmation of CLA infection in an additional five goat herds (Meldrum 1990; Lindsay and Lloyd 1991; Robins 1991). The goats on each of these premises were either slaughtered or placed under indefinite movement restrictions. In addition, a group of in-contact sheep was slaughtered, but the animals showed no lesions of CLA at necropsy.

In June 1991 *C. pseudotuberculosis* was identified in a sheep flock in the southwest of England (Robins 1991). Tracings from this outbreak led to the identification of a further six infected flocks of indigenous sheep breeds in southern England and a thorough investigation failed to establish any links between the infected animals and any recently

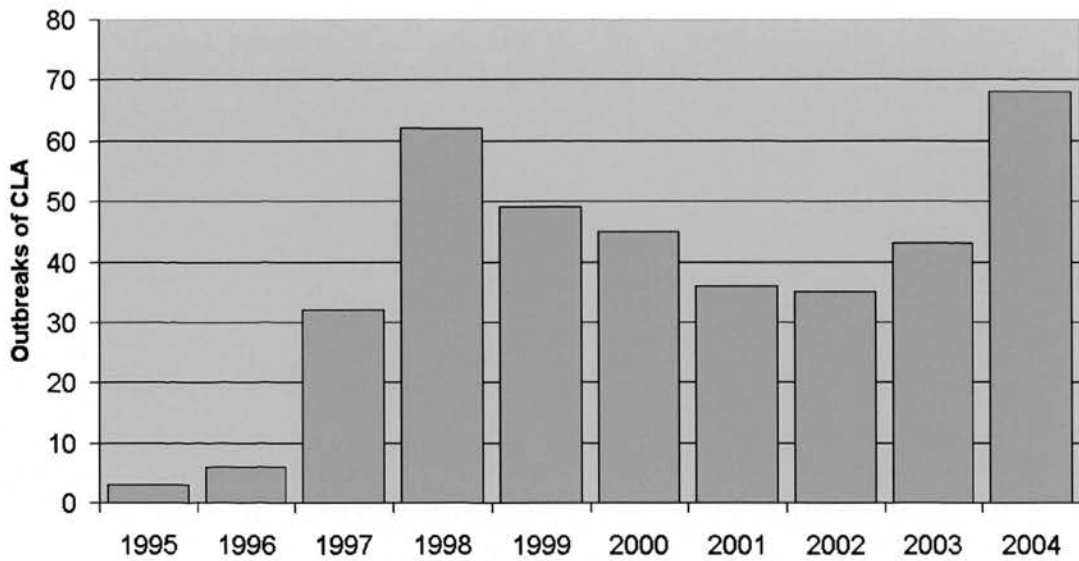
imported stock. At this point, after considering the extent of infection and the fact that CLA was present within most countries of the European Community, UK Government Ministers decided to deregulate the disease and all animal movement restriction orders still in place were suspended.

Over subsequent years the condition of caseous lymphadenitis was identified in sheep flocks and goat herds from other parts of England (Laven and others 1997; Rizvi and others 1997; Smith and others 1997; Watson and Preece 2001), Wales (Anon 2000) and Scotland (Anon 1996a; Scott and others 1997). The condition was then confirmed in N. Ireland (Malone and others 2002) and the Irish Republic (O'Doherty and others 2000; Bradshaw 2003) affecting animals recently imported from Scotland and England, respectively.

Examination of data produced through the Veterinary Investigation Diagnosis Analysis (VIDA) database (Anon 1996b, 2003b) gives some indication of how the disease spread within Britain after its initial diagnosis. The VIDA database records disease incidents on a yearly basis - an incident being defined as a single or multiple animals affected in one flock. A specific code number for ovine and caprine caseous lymphadenitis was provided in 1991, after which the diagnosis could be recorded by Veterinary Investigation Officers within the VIDA system.

Examination of VIDA records indicates that during the early part of the 1990's recorded incidents of CLA remained in low single figures. However the diagnostic rate began to rise rapidly in the second half of the decade, reaching a peak of 62 outbreaks in 1998 (see Figure 3.1). There was a subsequent reduction in the number of diagnoses to a low of 35 in 2002, partially attributable to the decline in submissions to Veterinary Investigation Centres following the foot and mouth disease epidemic of the previous year before rising to a figure of 68 for 2004.

Figure 3.1 Outbreaks of CLA in GB sheep flocks from 1995-2004 (Source VIDA 2005).



Whilst VIDA records may provide useful information on disease trends, it is generally acknowledged that this type of passive or scanning disease surveillance is subject to certain inherent biases (Hoinville and others 2009). An accurate assessment of disease prevalence may therefore require more active or targeted forms of surveillance.

Several authors have identified a need to establish more robust estimates of the prevalence of CLA infection in Great Britain and expressed concerns that the condition may have been under-reported within VIDA (Baird 1997; Laven and others 1997; Rizvi and others 1997; Smith and others 1997; Winter 1997). In this regard a number of small studies of ovine CLA have been conducted in the UK in recent years. A postal survey was organised in 2000 amongst members of the Sheep Veterinary Society and of three unnamed terminal sire Breed Societies. Recipients were questioned about their experiences of CLA between the years 1990 and 1999 (Binns and others 2002). In this study 18% of the 264 veterinary surgeons that responded, had encountered the condition in sheep at least once during the period. Approximately 45% of the sheep breeders had

seen abscesses within their flocks. However only a relatively few farmers (14%) had had these lesions investigated – although when this was done three quarters of cases were confirmed as CLA. Although this postal survey was acknowledged to suffer from a lack of statistical power (due to a low response rate), the authors did conclude that the prevalence and incidence of the disease in the UK was increasing.

The Veterinary Laboratories Agency (VLA) conducted two bacteriologically based surveys of CLA in sheep (Mechie 1998). In the first survey a free bacteriological screening service was established for farmers, operated through their private veterinary surgeons. In the second scheme, swabs from abscesses identified in sheep carcasses at meat inspection were submitted by the Meat Hygiene Service (MHS) to the VLA for culture. In each case the number of submissions was relatively low. Although several new flock outbreaks were identified through the farm-based survey (and recorded by VIDA), no cases of CLA were confirmed at the small number of meat plants that provided swabs (S. C. Mechie, personal communication).

Both “caseous lymphadenitis with emaciation” and “caseous lymphadenitis (generalised)” appear on a list of specified conditions drawn up by the MHS, which necessitate the total condemnation of an ovine or caprine carcass by meat inspectors (HMSO 1995; Grist 2006). Among sheep of all ages slaughtered for human consumption in Great Britain during 2004, lesions considered to be CLA were recorded at abattoirs on 598 occasions. This represented slightly more than 0.004% of the approximately 14.9 million sheep carcasses inspected (M. Gallego, personal communication). The condition was not identified in any of the approximately 8,000 goats slaughtered in Britain for human consumption over the same period. These figures would seem to suggest a very low prevalence of CLA within the British sheep population. However the majority of sheep carcasses examined by meat inspectors are prime lambs and therefore less than one-year-old at the point of slaughter. This has the effect of skewing the MHS statistics on CLA towards prevalence at the lower end of the age spectrum. It is recognised that younger sheep are proportionately less affected by

the disease (Nuttall 1988; Severini and others 2003; Paton and others 2005) so it is likely that the level of condemnation at abattoirs is not a true reflection of prevalence within the sector as a whole.

It had been established that the introduction of clinically or sub-clinically affected animals is the primary means by which CLA is spread between sheep flocks (Pepin and others 1994a). Consistently, reports in the United Kingdom have indicated that a disproportionate number of rams are affected by the disease (Rizvi and others 1997; Scott and others 1997; Watson and Preece 2001). Disease prevalence of up to 50% has been recorded in ram groups, whilst the disease has remained undiagnosed amongst the breeding ewes on the same holding. Indeed, in most flock outbreaks dealt with by SAC Veterinary Services, the first animals to be identified with CLA have been rams (Baird 2000). Since breeding rams are the animals most frequently sold or loaned between flocks, sub-clinical or unrecognised infection of males can make them a possible vector of disease between otherwise closed flocks. In one closely studied Scottish case, CLA was shown to have been transferred between two flocks following the temporary loan of one ram for a short period during the breeding season (Baird 2003).

As most lesions occur in the lymph nodes of the head and neck, the feeding of a daily concentrate ration to rams at a common trough is a possible risk factor. Fighting behaviour exhibited by rams might also favour the spread of infection through traumatic injury to the skin of the poll, nose and ears (Rizvi and others 1997). This mode of transmission is known to be important for the spread of CLA amongst goats (Williamson 2001).

During the initial emergence of CLA within the UK sheep industry during the 1990's, animals from the so-called terminal sire breeds formed the vast majority of reported outbreaks. These breeds, whose primary purpose is in cross-breeding to produce progeny with superior growth and carcass characteristics, include the main native terminal sire breed, the Suffolk, and the two most important continental breeds, the

Texel and Charollais. The VLA and SAC VS monthly reports and letters published in the *Veterinary Record* in the period between 1991 and 2003, confirm that the great majority of CLA outbreaks in the UK in pedigree flocks of these three breeds (Anon 1996a, 1997; Laven and others 1997; Rizvi and others 1997; Anon 2000, 2003a). At this time, when commercial flocks were affected by CLA, the index case was frequently identified as being a ram from one of these breeds (Scott and others 1997; Watson and Preece 2001). Anecdotal evidence from within the sheep industry also identified these breeds as being the worst affected by the condition – with the Charollais regarded by many as being the breed with the highest prevalence of CLA.

Although VIDA data and reports from other sources indicate an increase in the incidence of CLA, an estimate of national prevalence is difficult to reach. As an initial step to determining the significance of CLA to the sheep industry, a prevalence study was proposed within the terminal sire breeds. Because of the strategic position that this sector holds at the top of the so-called “breeding pyramid”, an assessment of this kind would provide useful information on the risks facing the wider UK commercial flock. By studying rams it was possible to further concentrate resources on the population that was considered to suffer from CLA most commonly. Since terminal sire rams are traded within and between different sectors of the industry they pose the greatest risk for spread of the infection.

In the mid-1990's Workers at the Dutch Central Veterinary Laboratory (ID-DLO) and Dutch Animal Health Service developed an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to the PLD exotoxin produced by *C. pseudotuberculosis* (Laak and others 1992). The test method was later modified to further improve specificity and sensitivity (Dercksen and others 2000). The assay is an indirect double antibody sandwich ELISA, employing PLD exotoxin purified from the supernatant of *C. pseudotuberculosis* cultures, and uses polyclonal rabbit anti-PLD serum as a capture antibody.

In a laboratory trial, which included sera from UK outbreaks of CLA, the calculated sensitivity of the modified ELISA when used in sheep was $79\% \pm 5\%$ and the specificity $99\% \pm 1\%$ (Dercksen and others 2000). The relatively low level of test sensitivity implies a significant number of false negative results in infected sheep. However, the high specificity of the test means that the likelihood of false positive results is low. As a consequence of this, the test is considered to be well suited for use in a disease prevalence study performed at a flock level.

The Maedi Visna (MV) Accreditation Scheme was originally established in the Great Britain by the State Veterinary Service (SVS) within the Ministry of Agriculture (Northern Ireland is designated MV free and consequently does not participate in the Accreditation Scheme). In 1995, testing and administration of the scheme was transferred from the SVS to the Scottish Agricultural College, under the auspices of the Sheep and Goat Health Schemes (SGHS). The MV Accreditation Scheme certifies qualifying sheep flocks as free from maedi visna virus infection, and goat herds as free from caprine arthritis and encephalitis virus (CAEV) infection. Accredited status within the Scheme is achieved and maintained on the basis of regular and targeted serological testing of flocks and herds.

In 2000 a total of 3,185 sheep flocks and goat herds were accredited within the MV/CAEV Accreditation Scheme - accounting for more than 180,000 individual animals. The predominant sheep breeds within the scheme at that time are shown in Table 3.1. The majority of member flocks are from the established terminal sire breeds. Because MV accreditation is a membership requirement of the respective Breed Societies, the majority of pedigree flocks in the terminal sire sector are also MV Scheme members.

**Table 3.1 Sheep and Goat Health Scheme member flocks by breed
(Source SGHS 2001).**

Predominant breed of Sheep	Number of member flocks
Texel	981
Suffolk	979
Charollais	379
Lleyn	126
Border Leicester	126
Rouge de l'Ouest	84
Bleu du Maine	70
Beltex	51

The basis of the MV Accreditation Scheme is the biennial testing of blood samples from a statistically significant number of animals in the member flocks. A total of approximately 68,000 blood samples are submitted for testing each year, from approximately 1,600 different sheep flocks. Because breeding rams are recognised as an important vector of infection in the epidemiology of MV, Scheme rules require that at each flock test, all the entire male sheep of 18-months or older are blood sampled. Thus blood samples originating from rams compose approximately 10% of the total annual submissions within the MV Accreditation Scheme. Since the majority of MV accredited flocks come from the terminal sire sector, a serological survey of samples from member flocks provides an effective cross-section of that part of the GB sheep industry. What follows is a description of a serologically based study of CLA prevalence in Great Britain employing blood samples taken as part of the MV accreditation scheme, which was planned and co-ordinated by the author.

Materials and methods

Sample collection was carried out during a period of nine months between March and November 2000. All MV Accreditation Scheme members submitting blood samples for testing were asked if, on completion of MV testing, the submitted blood samples might

be used for additional anonymised research purposes, to which no member objected. Submitted blood samples were received at the SAC Veterinary Services laboratories in Dumfries, Edinburgh, Aberdeen and Inverness. At each laboratory serum was collected from these blood samples taken from male animals within each batch of sampled animals and stored at -20°C. The frozen sera were sent to the Dutch Central Veterinary Institute (ID-DLO) at Lelystad, in the Netherlands.

At the ID-DLO these sera were tested blind using the modified ELISA method described in detail by Dercksen and others (2000). Thus, test results were expressed as net extinction values (the extinction value with the positive antigen minus the extinction value with the negative antigen). Values below 100 were considered negative, values of 100-200 were considered inconclusive and values above 200 were considered positive. When sera gave an inconclusive test reaction in the ELISA, a further Western blot technique was applied (Kamp and others 2001). The Western blot result was taken as positive when at least two bands were clearly visible on the test membrane at 31 kDa and 68 kDa. If the Western blot gave a negative result (no bands), or an unconvincing result (one or two very weak bands) the sample was classified as inconclusive.

Results

A total of 2,538 serum samples from rams were tested. These samples originated from a total of 745 different GB flocks. From the sera tested, a total of 244 samples produced net extinction values in the ELISA test that fell above the positive threshold. In a further six cases, the initial ELISA net extinction values fell within the inconclusive range, but were followed by a positive result in the Western blot assay. Thus, from the 2,538 serum samples tested, a total of 250 were considered positive for PLD antibody. This gave an overall *apparent prevalence* of seropositive animals within the sample group of 9.93% (with 95% confidence interval of 8.76% to 11.1%; 99% confidence interval of 8.39% to 11.46%).

A total of 134 flocks had one or more seropositive animals, this being just under 18% of the flocks sampled. In a total of 48 (or 36%) of these seropositive flocks, there was more than one ELISA-positive animal. Five or more seropositive animals were detected in the same flock submission on ten occasions.

In Figure 3.2 the geographical locations of those flocks with seropositive rams are indicated on a county or regional basis. In Figure 3.3 the number of submitting flocks that contained seropositive rams is related to the same geographical information. In Figure 3.4 the proportion of submitting flocks that contain seropositive rams is indicated for each respective region.

Figure 3.5 indicates the relative proportion of the different sheep breeds amongst the 134 flocks containing seropositive rams. Those flocks recorded in the "mixed breed" category are composed of animals from two or more different sheep breeds. Those flocks in the "Suff/Char/Tex" category are composed of sheep from two or more of the Texel, Charollais and Suffolk breeds only.

Figure 3.2 Shaded areas indicate region or county in which flocks containing seropositive rams are located.

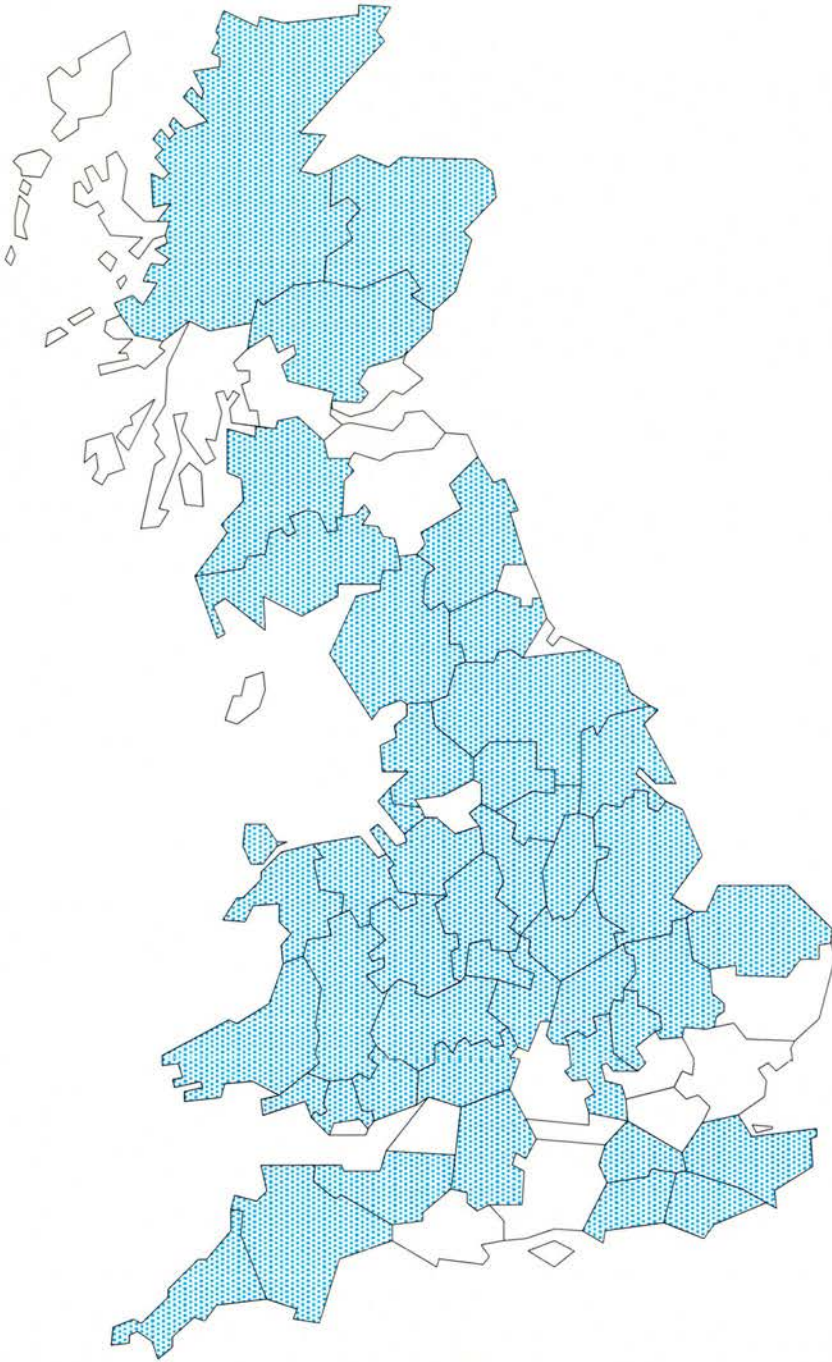


Figure 3.3 Total number of sheep flocks containing seropositive rams identified by county or region.

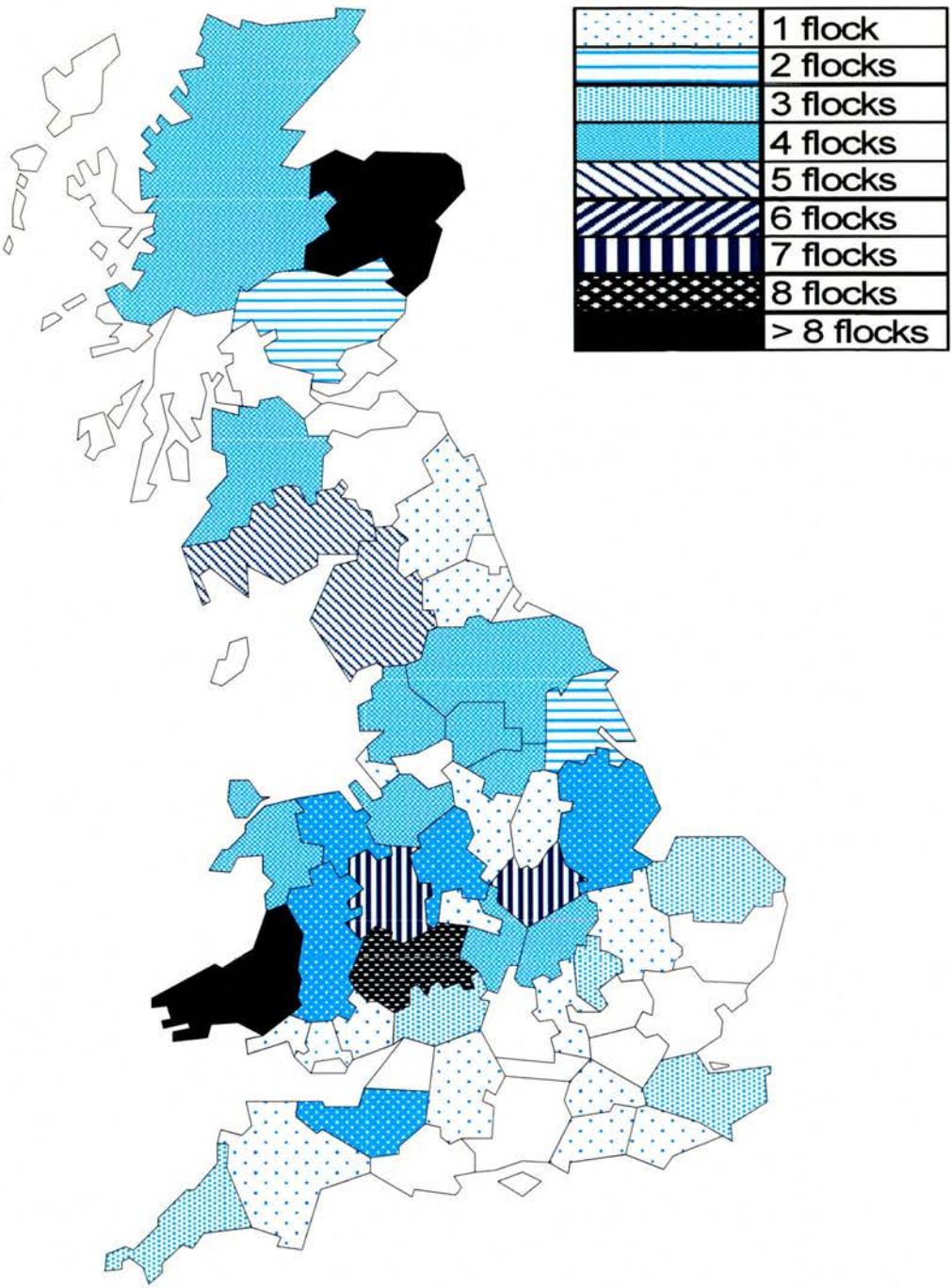


Figure 3.4 Percentage of submitting sheep flocks containing seropositive rams identified by county or region.

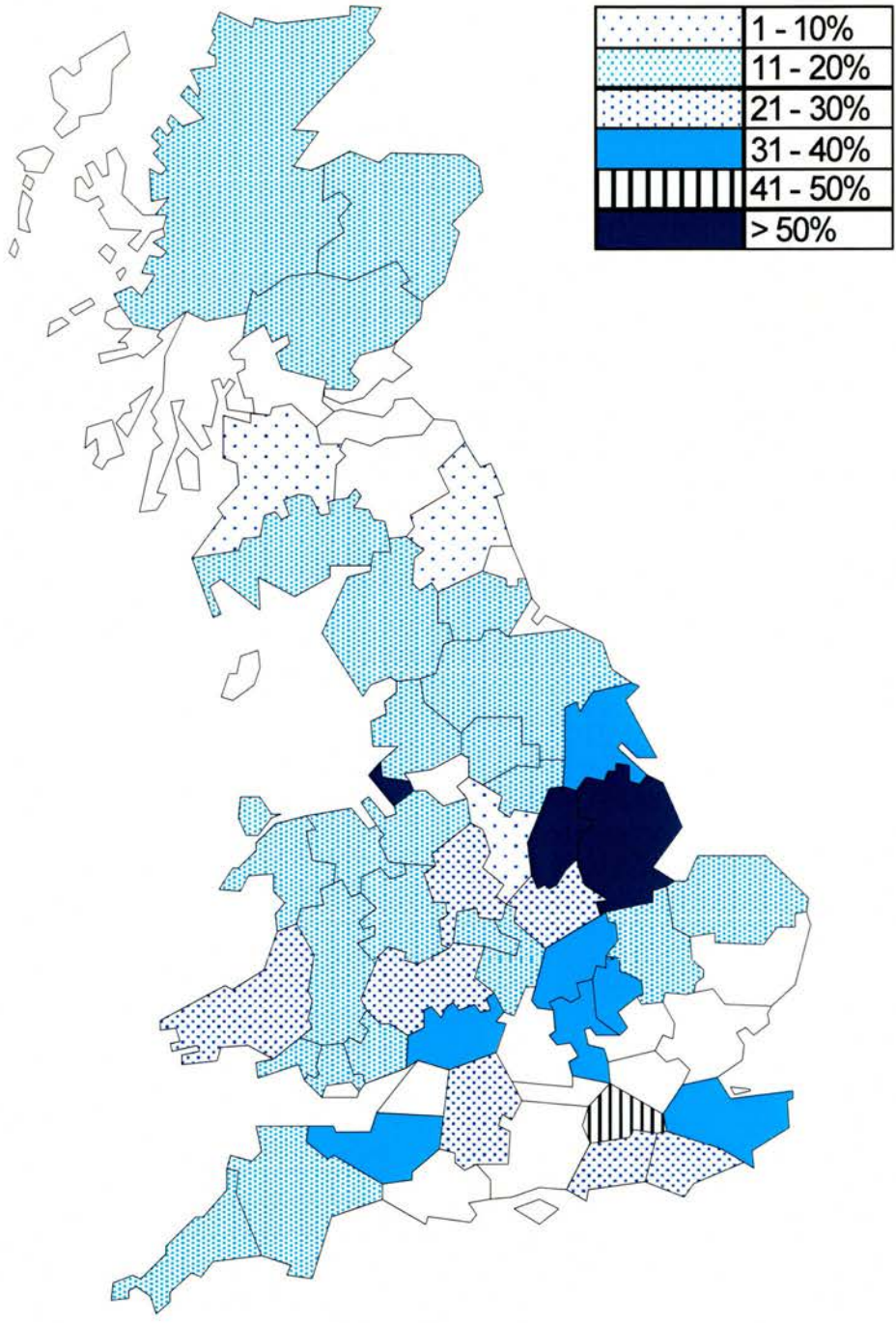


Figure 3.5 Breed of sheep flock containing seropositive rams.

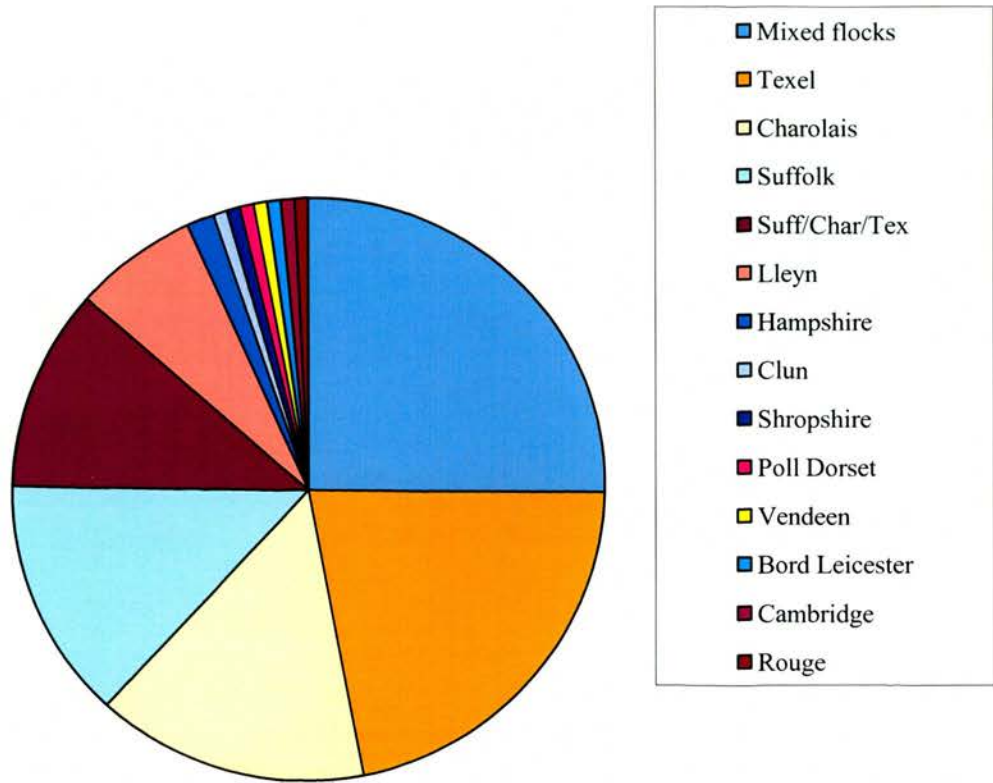


Table 3.2 Single breed flocks containing CLA antibody positive rams.

Breed	Total No. of seropositive flocks	Proportion of the seropositive flocks of single breed (85) %	Proportion of SGHS Membership (1999 figures) %
Texel	29	34	31
Charollais	20	24	12
Suffolk	18	21	31
Lleyl	9	10	4

A total of 85 flocks containing seropositive rams were composed of a single breed of sheep. The number of positive flocks from the four breeds that gave most positive results is recorded in Table 3.2. These figures are also given in terms of the total number of single breed seropositive flocks. Table 3.2 also includes a figure for the proportion that each breed constitutes within the total membership of the MV Accreditation Scheme. Seropositive flocks from the other breeds were in such small numbers as to make this type of calculation meaningless.

Discussion

In this study the population from which sera were collected, namely member flocks of the MV Accreditation Scheme, represents a relatively small sector of the UK sheep industry. In examining sera from 745 individual sheep flocks, approximately 0.95% of the 77,900 sheep units in the UK national flock at that time (Templeton 2001) were sampled. By the very nature of the MV Scheme, the sampled flocks are almost exclusively purebred and predominantly from the pedigree terminal sire breeds. Further to this, by examining only sera from rams within the member flocks, further bias has been added to the sampling.

Whilst acknowledging the limitations of the sampling technique, the identification of seropositive animals in some 18% of the tested flocks was greater than anticipated. Published data from VIDA has indicated an increase in the number of CLA outbreaks in Britain. However, information on the particular sheep breeds affected in these outbreaks and on their location is not easily accessible. This study confirms that at the time the condition was both geographically widespread within Britain and present within a broad range of strategically important sheep breeds.

The highly specific nature of the ELISA test, published as 99% +/- 1%, indicates that few false positive results are likely within the data set. However, the more modest

sensitivity of the assay suggests that a proportion of the negative results may in reality come from animals infected with *C. pseudotuberculosis*. Based on the published sensitivity of 79% +/- 5%, the overall flock prevalence of 18% indicated in this study may in reality have been an underestimate of the real situation.

The findings from this study are broadly in line with the consensus of published reports of CLA within GB and are also consistent with much of the anecdotal evidence from veterinary practitioners and farmers. In particular the apparently higher rate of seroprevalence amongst Charollais compared to other terminal sire flocks provides some support for the perception within the sheep industry that this breed is badly affected by CLA. The results are also of significance in terms of disease control. The wide geographical distribution of the infected flocks suggests that there are few if any areas of the country with freedom from disease.

Examination of the geographical data (Figure 3.2) indicates that infected flocks were detected in most regions of Britain. In this study the largest numbers of seropositive flocks were found in the Grampian Region of Scotland and in Dyfed in Wales, with 12 and 11 positive flocks respectively (see Figure 3.3). However, this was biased by a particularly high SGHS flock membership rate in both those regions. In Figure 3.4 where seropositive flocks are represented as a proportion of total submissions, both Grampian and Dyfed are seen to have a flock prevalence at a similar level to most other regions in Britain. There are however four regions where the proportion of flocks that were seropositive was greater than 40%. This finding is also slightly misleading, since in each case the total number of submitting flocks from that county or region was very small. Samples from only one flock were submitted from both Merseyside and Nottinghamshire, and on each occasion CLA positive samples were detected. One of the two flocks submitting from Surrey was found to be seropositive for CLA. Perhaps more significant is the fact that four out of the seven flocks sampled in Lincolnshire contained seropositive rams.

No seropositive flocks were recorded in a number of English and Scottish regions. In some cases this may be explained simply by an absence of MV Scheme member flocks in those areas – for example in Greater London and the Western Isles of Scotland. However, this cannot be used to explain the apparent lack of infection in regions such as the Scottish Borders and the Lothians. Membership of the MV Scheme is reasonably strong in those areas and a significant number of flocks did submit sera during the study period. The author's personal knowledge of sheep flocks in these areas also indicates that CLA is indeed present there. The conclusion therefore must be that these findings do reflect a lower overall disease prevalence there.

The results represented in Figure 3.5 indicate that in total 61% of the seropositive flocks were composed of sheep from one or more of three particular sheep breeds - Texel, Charollais and Suffolk. It should also be noted that animals from these breeds were present in other seropositive flocks where a number of different breeds were run together. Indeed only 34 of the 134 seropositive flocks contained no animals from these three breeds. Again the composition of the MV Accreditation Scheme is of relevance in this regard. As Table 3.2 indicates, the finding is less significant than it might at first appear, since Suffolk, Texel and Charollais flocks make up approximately 70% of the scheme members. Thus, it can be appreciated that although the Texel breed made up 34% of seropositive flocks composed of a single breed, it constitutes fully 31% of the membership of the SGHS. More interesting is the fact that the Charollais breed which makes up 12% of MV Scheme membership produced 24% of the single breed seropositive category. Also of note is the Welsh Lleyn breed that comprises only some 4% of the SGHS membership but which produced 10% of the single breed seropositive total. Of the 34 flocks in which none of the three major breeds (Suffolk, Texel and Charollais) were represented, nine were composed purely of Lleyn animals and a further six were composed of multiple breeds including Lleyn sheep. Many of the information forms completed by farmers when submitting blood samples for testing in the MV Accreditation Scheme give only animal identification numbers and no specific breed information. In such cases identifying the origins of a particular serum sample from a

flock containing more than one sheep breed is therefore impossible. Because of this lack of data, it is not possible to ascribe statistical significance to the breed-related findings.

One possible complicating factor in this study that should be considered is the effect that vaccination may have had on the results. Two regulated sources of CLA vaccine were available during the period of the study. Firstly, a number of shepherds who were aware of CLA infection within their flock had sought official approval for the manufacture and use of an "autogenous" vaccine. To enable such a vaccine to be produced, specific approval must be given by the Veterinary Medicines Directorate (VMD) within DEFRA. This is done by way of a Autogenous Vaccine Authorisation (AVA) (VMD 2009a). The bacteria required for the production of these vaccines are obtained directly from infected flocks – either through the submission to the manufacturing laboratory of swabs from CLA lesions or sub-cultures of *C. pseudotuberculosis* isolates from VI Centres. The manufacturing laboratory then produces a formalin-inactivated, whole cell vaccine, which may only be used in the flock of origin. An AVA is valid for one year and must therefore be renewed annually, on application to the VMD, if the owner wishes to continue using the vaccine. Consultations with the VMD indicate that an AVA equivalent (known at the time as a Special Treatment Authorisation) was granted for the production of autogenous CLA vaccine on 15 occasions during the year 2000-01. No further information on the location and type of flocks using this vaccine is available.

Since autogenous vaccine production employs a crude bacterin of unwashed cells, it must be assumed that small traces of PLD exotoxin are present within the prepared vaccine. As a result, an antibody response to PLD is likely to be elicited in the animals receiving the autogenous vaccine. The significance of this in relation to this particular study is the question of whether prior vaccination of sheep with an autogenous CLA vaccine may have led to a positive ELISA test. There is no published data on the use of the Dutch ELISA with sera from sheep that have received autogenous CLA vaccine. However in one published study from the United States the use of an equivalent bacterin

to inoculate lambs did not elicit a detectable antibody response (Leamaster and others 1987). A similar finding is described in Chapter 7, when a PLD ELISA assay was used to test sheep receiving an autogenous CLA vaccine and reactivity was equivalent to that of non-immunised control animals.

The second type of CLA vaccine available in the UK is that manufactured commercially by pharmaceutical companies overseas. No such CLA vaccine product is currently licensed for use in the United Kingdom. However some three flocks in England were granted what was then known as an Emergency Product Licence (EPL) by the Veterinary Medicines Directorate in the late 1990's. These twelve-month licences allowed for the importation and use of a CLA vaccine GlanvacTM manufactured in Australia by Commonwealth Serum Laboratories. However the Veterinary Medicines Directorate ceased issuing Emergency licences for GlanvacTM shortly afterwards, citing concerns that importation under EPL legislation might discourage an application for full product licence in the UK. More recent anecdotal evidence suggests that some sheep farmers are privately importing GlanvacTM into the UK without the required Special Treatment Certificate (VMD 2009b), although the use of such CLA vaccine obtained from overseas sources, which do not carry a relevant UK licence remains illegal.

GlanvacTM and similar products are toxoid vaccines which stimulate a highly specific immune response to the PLD exotoxin produced by the *C. pseudotuberculosis* organism (Paton and others 2003). Sera taken from animals that are known to have been vaccinated with GlanvacTM has not been tested with the ELISA used in this study. However, since PLD is highly conserved, it is likely that prior use of this proprietary CLA vaccine would lead to a positive test result in the Dutch ELISA. Again unpublished data based on the use of a similar ELISA supports this hypothesis (M. C. Fontaine, personal communication). It is therefore possible that the unregulated use of imported vaccine may have led to a number of positive results detected within this study.

It is therefore important to consider the likely effects of vaccine use on the results of this current study. By definition, an autogenous vaccine is used only in a flock that is already infected by disease. It is clear that in any event only a small number of flocks in the UK have used an autogenous vaccine against CLA. Similarly, although the private importation of CLA vaccine is feasible, it is acknowledged to be an expensive exercise. It is therefore likely to be undertaken only by farmers who are aware of an existing CLA problem within their flocks. By its nature the unregulated use of imported commercial vaccine is impossible to quantify, but it also seems unlikely that many flock-masters would have gone down that route. In addition, anecdotal reports suggest that the importation of proprietary CLA vaccines has only recently become widespread, but that it was at a much lower level in the period 1999-2000.

Thus the use of either autogenous or proprietary vaccines may conceivably have had the effect of increasing the total number of seropositive rams, but is unlikely to have increased the number of flocks containing seropositive animals. Therefore, although the confounding effect of vaccination may have played some part in artificially increasing the calculated seroprevalence in this study, the overall effect is considered to be negligible.

When the results of this study are applied to a model of infection in which CLA is commonly introduced to naive flocks by sub-clinically infected breeding replacements, the potential for further spread within the UK appears to be considerable. A high prevalence rate within the terminal sire breeds, coupled with the tendency for rams to be disproportionately affected by the disease, suggests that the spread of infection will be accelerated by widespread distribution of these sires through the rest of the sheep industry. In the UK the majority of rams are bought at a relatively small number of high profile ram sales. These attract consignors and buyers from outwith the immediate region, with the result that purchased rams may be transported very large distances. Analysis of the seropositive flocks in this study indicates that all the dominant terminal sire breeds are affected by the infection and that disease prevention is unlikely to be

achieved by purchasers through the selection of one breed over another. The commercial sector of the industry, which is the recipient of the majority of sale rams, must therefore be at great risk of buying-in infection in the form of sub-clinically affected animals.

The results of this study indicate a need to raise awareness of the disease within both the pedigree and commercial sectors and emphasise the urgent requirement for a reliable means of controlling the spread of infection. The data, whilst describing the extent of infection within one important sector of the industry, also provides an encouragement for a larger scale prevalence study within the wider sheep industry.

Chapter 4: THE EFFECTS OF COMMERCIAL DAIRY PASTEURIZATION ON THE SURVIVAL OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* IN GOATS' MILK

Summary

To test the ability of *C. pseudotuberculosis* to survive conventional pasteurization methods, goat's milk that had been inoculated with the bacterium was cultured after being passed through a pasteurization plant. Batches of ultra heat-treated (UHT) goat's milk were inoculated with *C. pseudotuberculosis* to concentrations of approximately 10^2 , 10^3 , 10^4 and 10^5 colony forming units (cfu) per millilitre of milk. In order to simulate natural caseous lymphadenitis infection, a further batch of milk was inoculated with a solution containing purulent discharge recovered from a caseous lymphadenitis lesion. Each batch of inoculated milk was passed through a pilot pasteurization plant used in milk hygiene research and equivalent in characteristics to full-scale commercial pasteurization equipment.

Conditions used in pasteurization during this experiment were equivalent to the UK legal minimum, in which the milk was rapidly heated to 72°C and held at that temperature for 15 seconds, before being cooled. Twenty-four hours after pasteurization, samples of each batch of milk were inoculated directly onto blood agar and incubated at 37°C for 48 hours. In a process of enrichment, separate samples of the pasteurized milk were placed in a brain heart infusion broth and maintained at 37°C for 72 hours, before aliquots were used to inoculate blood agar, which was incubated for a further 48 hours. Any bacterial colonies noted on the blood agar after incubation were differentiated using standard microbiological techniques. No growth of *C. pseudotuberculosis* was identified on culture of samples from any batch of pasteurized milk. It is therefore concluded that standard commercial pasteurization techniques are effective in killing *C. pseudotuberculosis* in milk.

Introduction

The visceral form of *C. pseudotuberculosis* infection in domestic animals can include the development of lesions within the mammary gland. Although occasionally recorded in other parts of the world (Adekeye and others 1980; Hommez and others 1999), mastitis in dairy cows due to *C. pseudotuberculosis* has been identified as a problem of particular importance in Israel (Yeruham and others 1997). The condition is prevalent in many Israeli dairy herds, where outbreaks may appear sporadically or in epidemic form. (Yeruham and others 2003a). The milk production of affected quarters decreases and may cease completely, while somatic cell counts increase dramatically. The organism is readily isolated from the milk of mammary glands suffering acute, chronic and subacute forms of the disease. In general the condition in dairy cows responds poorly to antibiotic treatment (Shpigel and others 1993). Similar mastitis has been induced in dairy cows by the inoculation of as few as 2,000 colony forming units (cfu) of *C. pseudotuberculosis* into the teat canal (Aroch and others 2003).

A primary mastitis of the form recognised in cattle has also been reported in sheep and goats: however, in those species it is recorded very infrequently (Brown and Olander 1987). A rather more common form of infection in goats and sheep is abscessation of the mammary gland (Figure 4.1), with abscesses forming following a primary mastitis, or more often as the result of extension from an adjacent foci of infection (Burrell 1981). Frequently the site of this initial infection is one or both of the supramammary lymph nodes. Rather than these lesions progressing to discharge their purulent contents externally, they can rupture internally, with the release of infected material into the mammary tissue itself. One or more foci of infection are then established within the udder itself. In such cases the organism is easily identified in milk withdrawn from the affected quarter (Schreuder and others 1990).

Figure 4.1 Mammary gland abscess due to *C. pseudotuberculosis* infection in an adult ewe (picture taken by author).



The first published case of human infection by *C. pseudotuberculosis* was reported in 1966 (Lopez and others 1966). Subsequently, most reported zoonotic infections have been associated with direct contact with clinically affected animals by farmers, shearers or abattoir workers (Peel and others 1997). Significantly, however, one recorded case of human infection has been associated with the consumption of raw goat's milk by a city resident with no other reported direct or indirect contact with farm animals (Goldberger and others 1981).

Potential contamination of milk by pus from CLA lesions has been a concern in the dairy goat industry of the Netherlands, where an eradication and control scheme was established to reduce the risks (Dercksen and others 1996). As CLA has emerged as a significant disease within the goat industry of the United Kingdom, producers that are anxious to ensure the quality of their product have expressed similar concerns. In particular, assurances have been sought that the process of pasteurization currently

employed in dairies is sufficient to remove all viable *C. pseudotuberculosis* organisms from milk for sale.

Most commercial milk pasteurization in the UK is carried out using the High Temperature Short Time (HTST) method, where continuously flowing milk is heated to a temperature of 71.7°C or above. It is held at this temperature for a minimum of 15 seconds and then immediately cooled to a temperature not more than 10°C. This procedure is designed to ensure the microbiological safety of the milk and to enhance its effective shelf life.

The routine pasteurization of milk was originally established to remove the risk of potential zoonotic infection by *Mycobacterium bovis*, the cause of bovine tuberculosis. Although the majority of pathogenic and spoilage bacteria are rendered non-viable by this process, a small number of so-called thermophilic organisms are capable of surviving conventional pasteurization techniques. The types of bacteria most commonly recognized as possessing this ability are the spore-forming *Bacillus* genus and the coryneform group of bacterial organisms (Muir 1990).

In recent years, concern has focused on the possibility that *Mycobacterium avium* ssp. *paratuberculosis* (*Map*), the cause of Johne's disease in cattle and other species, may survive dairy pasteurization and remain viable in commercial milk supplies (Grant and others 2005). The factors influencing this resistance to heat treatment are not well understood, but may include the number of *Map* present within the milk, the tendency of the organism to form large clumps in solution, and the protection from heat afforded by the thick mycolic acid layer around the cell (Klijn and others 2001; Grant and others 2005). *C. pseudotuberculosis* is known to possess important characteristics in common with the mycobacteria. It has been suggested that the similar waxy cell wall mycolic acid enables the organism to survive for extended periods within the environment (West and others 2002), a feature common to other members of the actinomycetaceae. The

tendency of the organism to form clumps in solution is another feature that could enhance the thermoduric properties of *C. pseudotuberculosis*.

Objectives of this study

Under certain circumstances, viable *C. pseudotuberculosis* organisms may appear in milk destined for human consumption and produced by cattle, goats or sheep. On this basis the author considered it important to establish whether the normal process of commercial pasteurization would kill *C. pseudotuberculosis* present in otherwise sterile milk samples. As part of this objective, any potentially protective effect resulting from the clumping of *C. pseudotuberculosis* was assessed by increasing the numbers of bacteria used to inoculate milk samples in log-fold increments. In addition, any thermo-protective effect resulting from the encasement of *C. pseudotuberculosis* within pus was assessed by inoculating milk directly with the contents of active CLA lesions.

Materials and methods

The following experimental procedure was performed on two different occasions. *C. pseudotuberculosis* strain 3/99-5 was passaged three times on blood agar base (Oxoid, Basingstoke, Hampshire, England) supplemented with 5 % (v/v) of citrated sheep blood (BA), at 37°C for 48 hours. Anti-microbial sensitivity testing of the isolate was performed in the established manner by the use of antibiotic infused discs (Mast Diagnostics, Merseyside, U.K.) establishing a resistance in the isolate to streptomycin sulphate. A growth of *C. pseudotuberculosis* strain 3/99-5 was then suspended in phosphate buffered saline (PBS) and the concentration of organisms determined using an ATB Densimat densitometer (bioMérieux, Basingstoke, UK) and the McFarland standard (Quinn and others 1994b). This solution was then used to inoculate 20 litre batches of commercially purchased ultra heat-treated (UHT) goat's milk, to a concentration of approximately 10^2 , 10^3 , 10^4 and 10^5 cfu/ml of milk.

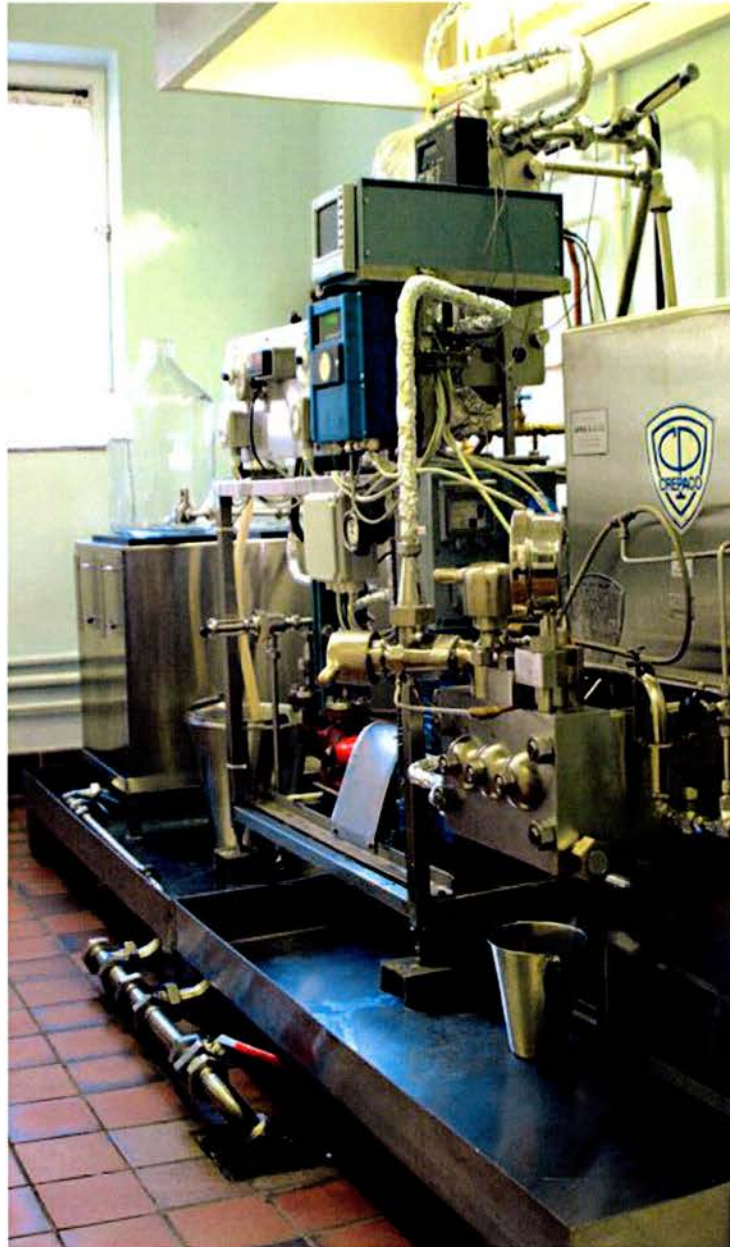
A fifth batch of UHT goat's milk was inoculated with a solution of PBS containing pus recovered from a natural CLA infection – this being an ovine parotid gland abscess for the first experimental run and a caprine udder abscess for the second. This less quantitative approach of introducing a purulent discharge meant that the final effective concentration of *C. pseudotuberculosis* organisms within the milk was significantly different between the two experimental runs. During the first experiment the addition of purulent discharge produced a final concentration of organisms within the milk of approximately 6×10^3 cfu/ml, while on the second occasion a concentration of 8×10^4 cfu/ml was achieved.

Each inoculated batch of UHT milk was passed through a pilot pasteurization plant (Figure 4.2) comparable in characteristics to full-scale commercial equipment. This plant was located at the Hannah Dairy Research Institute, Ayr, Scotland where it was constructed for the purposes of milk hygiene related research and operated in line with industry standards. The heat exchanger plate pack, holding sections, temperature, pressure and flow monitoring probes and logging equipment were externally accredited by Honeywell Control Systems Ltd. (Glasgow, UK) and Pressure Technical Services Ltd. (Manchester, UK), respectively. For this experiment, the pasteurizing plant was configured such that homogenization was applied upstream of heat-treatment as in a conventional industrial plant. The pumping speed, and hence flow rate through the heat exchanger was controlled such that the milk was held at 72°C for 15 seconds before being rapidly cooled.

After the pasteurization plant was brought up to the correct temperature and the flow rate stabilised for the specified conditions, the five batches of inoculated milk were introduced to the plant in succession. The milk sample containing the lowest number of organisms (10^2 cfu/ml) was pasteurized first. Water was then passed through the plant to provide a visible break before the next batch of milk. Subsequent batches of milk containing progressively higher concentrations of bacteria were passed through the machine in the same way, with the milk inoculated with pus in PBS being processed last.

During the pasteurization of each batch, five separate 500 ml samples of the pasteurized milk were collected. These samples were immediately cooled on ice and then refrigerated for 72 hours at 4°C. This period of refrigeration was included to allow for the recovery of any heat-damaged but viable bacteria, before the commencement of bacterial culture.

Figure 4.2 Pilot pasteurisation plant used during this experiment at Hannah Dairy Research Institute, Ayr (picture taken by author).



Five separate 200 µl aliquots from each of the 500 ml samples of pasteurized milk were used to inoculate blood agar plates (BA; Oxoid, Basingstoke, Hampshire, England), which were then incubated for 48 hours at 37°C. To enhance detection of viable bacteria within pasteurized milk, 100 ml of each 500 ml pasteurized sample was removed and supplemented with 800 ml of brain heart infusion broth (BHI; Oxoid, Basingstoke, Hampshire, England). To reduce the potential effects of bacterial contamination, streptomycin sulphate was included within the broth at a concentration of 5 µg/ml. Incubation at 37°C and shaking was then performed for a period of 72 hours, after which five 200 µl aliquots of the BHI/milk mixtures were inoculated onto BA plates and incubated for 48 hours at 37°C. Any bacterial colonies observed on BA plates after incubation were differentiated using standard microbiological techniques, including observations of colony morphology, Gram-staining and microscopic analysis.

As a positive control, a 10 ml sample from each 20 litre batch of milk was retained after the milk had been inoculated, but prior to pasteurization. Five 200 µl aliquots of each retained milk sample were inoculated onto BA plates and incubated as above. Bacterial colonies observed after incubation were again differentiated using standard microbiological techniques. In addition, the API Coryne diagnostic test kit (bioMérieux, Basingstoke, UK) was used, according to the manufacturer's supplied instructions, to positively identify bacteria presumptively identified as *C. pseudotuberculosis*.

Results

For each of the milk samples inoculated with 10^2 , 10^3 , 10^4 or 10^5 cfu/ml, or pus from a CLA lesion, no growth of *C. pseudotuberculosis* was evident following pasteurization, either on direct culture of the milk samples or on culture of pasteurized samples supplemented with BHI. In addition, there was no growth of *C. pseudotuberculosis* following culture of any sample following enrichment in BHI broth. In contrast, the culture of samples of the inoculated milk taken prior to pasteurization (positive control samples) produced a moderate to heavy bacterial growth in all cases. These bacterial

colonies were subsequently positively identified as *C. pseudotuberculosis* using API Coryne test, confirming the viability of the initial inoculum.

Discussion

In the duplicate experiments conducted, there was no evidence that *C. pseudotuberculosis* in milk could survive pasteurization using conditions equivalent to the statutory minimum for milk sold in the UK, *i.e.* heating to a temperature of 71.7°C for at least 15 seconds. Not only was this the case for milk inoculated with bacterial cultures prepared in the laboratory, but also for milk containing the purulent discharge of CLA lesions. This latter situation was felt to most closely replicate natural infection in dairy cattle, goats and sheep, where the organism enters the milk secondary to mastitis or following the extension of infection from an abscessed lymph node.

Where milk samples were inoculated with laboratory cultured organisms suspended in saline, bacteria were likely to be present as single cells and more frequently in larger cell clumps. In contrast, where purulent discharge from CLA lesions was used for inoculating the milk, a proportion of the *C. pseudotuberculosis* organisms were likely to have been located intracellularly, within monocytes and macrophages (Pepin and others 1994b). In addition, these would have been surrounded by a matrix of living and dead leucocytes, cellular debris and serum. While this additional coating might have been thought to provide the organisms with a further degree of protection during the pasteurization, the results of this experiment indicate that this was not the case.

These results therefore confirm that the current statutory requirements for pasteurization in the UK are effective in protecting public health. In reality, since 1998 the majority of commercial dairies processing goat's milk for the retail trade employ heating at 72°C for a total of 25 seconds, rather than the legal minimum of 15 seconds. This is largely on the insistence of major supermarket clients, in response to the perceived risks of *Map* survival within pasteurized milk products. Clearly, this extended heating time has the

effect of reducing still further any possibility that *C. pseudotuberculosis* organisms might survive the pasteurization process.

Certain other pasteurization processes are currently employed within the sheep and goat dairy industry. These include batch pasteurization of milk at temperatures of 83°C for 15 minutes, in the production of yoghurts and fromage frais. For the production of ice cream, a temperature of 65.6°C is maintained for 30 minutes. In each of these cases the potential for the survival in milk of *C. pseudotuberculosis* is negligible. However, cheese manufacturers may use a process of thermising, which reduces the number of viable bacteria within the milk by heating to 65°C for 15 seconds in a continuous flow pasteurization plant. Others use raw milk from sheep and goats as the basis for cheese making. A laboratory study that simulated commercial cheddar cheese-making using raw bovine milk, established that *Map* was concentrated 10-fold in the production process (Donaghy and others 2004). Depending upon the type of cheese being produced, bacterial survival can vary with moisture content, pH, salt content, temperature and the antagonistic effects of starter bacteria and other organisms. It is by no means certain therefore, that any *C. pseudotuberculosis* that may be present within raw milk for cheese manufacture, would survive in sufficient numbers in the finished product to represent a potential risk to human health. However, in the absence of published findings in this area, it would be sensible for producers of such products to be aware of the possible risks presented by CLA and to take steps to control the infection in milking animals.

Chapter 5: CONTROL OF CASEOUS LYMPHADENITIS IN SIX LOWLAND SHEEP FLOCKS BASED ON CLINICAL EXAMINATION AND REGULAR ELISA TESTING.

Summary

In an effort to control the spread of infection within a number of CLA-affected flocks, sheep on six holdings were serologically tested at regular intervals using an antibody ELISA with a specificity of 99% +/- 1% and a sensitivity of 79% +/- 5%. Western blot assays that detected antibodies to the PLD exotoxin of *C. pseudotuberculosis* were used as a further test for ELISA inconclusive results. Owners were advised that any sheep demonstrating clinical signs of CLA or testing positive for PLD by ELISA and/or Western blot be removed from the flock.

Of the six trial flocks, one was dispersed after only two blood tests and in another the recommendations for CLA control were not followed and infected animals were retained within the flock. In the remaining four flocks the application of the testing regime and other advice enabled the disease to be controlled to such an extent that the appearance of new clinical cases of CLA was effectively halted, this remaining the case for up to five years after the end of the trial. In two of these flocks a small number of seropositive animals were detected at the end of the process. However, on the other two holdings all sheep were seronegative in the final two flock tests, consistent with the complete eradication of infection. On this basis it is suggested that a test and cull programme may be considered a legitimate and effective form of disease control in some flocks affected by CLA.

Introduction

Due to the high level of sub-clinical CLA infection in sheep, efforts at removing those individuals showing external signs of disease are ultimately unable to establish control of the disease. Serological tests based on the detection of antibody to PLD have been

explored as a means of controlling the disease in sheep and goats through the identification and removal of infected carrier animals (Sutherland and others 1987; Menzies and Muckle 1989; Muckle and others 1992; Ter Laak and others 1992). Most promising in this respect was felt to be the development of enzyme-linked immunosorbent assay (ELISA) methods (Menzies and others 1994).

Initially the antigens employed in CLA-diagnostic ELISAs were preparations of *C. pseudotuberculosis* cell wall or exotoxin derived from culture supernatants (Maki and others 1985; Sutherland and others 1987; Kuria and Holstad 1989b; Ellis and others 1990; Sting and others 1998). In the majority of these assays, test sensitivity was good but specificity was relatively poor, reflecting potential cross-reactions with extraneous protein molecules present within the antigen preparations. The developers of more recent ELISAs have claimed specificity and sensitivity levels that would be sufficient to allow their use as diagnostic tools in the field. Improvements have included the use in ELISAs of purer sources of PLD antigen, such as that derived by recombinant technology from *E. coli* containing a plasmid bearing the *pld* gene (Menzies and others 1994). In that paper the assay had a reported test sensitivity of 86.3% and the specificity of 82.1%. Another ELISA method was reported to have test specificity of 100%, with the sensitivity of detection of total antibody being 71% (95% confidence interval 63% – 78%), and the sensitivity of detection of IgG antibody to *C. pseudotuberculosis* being 83% (95% confidence interval 76% – 89%) (Binns and others 2007). The sensitivity of the IgG antibody assay was believed to be higher because of the greater affinity of IgG class antibodies compared with the IgM antibodies also detected by the total antibody ELISA. However, the ELISA coming closest to the requirements of a diagnostic test was developed in the Netherlands (Ter Laak and others 1992) and was later refined to further improve specificity and sensitivity (Dercksen and others 2000). This assay is an indirect double antibody sandwich ELISA, employing PLD exotoxin purified from the supernatant of *C. pseudotuberculosis* cultures, and uses polyclonal rabbit anti-PLD serum as a capture antibody. The improved method is calculated to have a specificity of 99% +/- 1%, and a sensitivity of 79% +/- 5% in sheep. The very high specificity of this

ELISA, but the relatively low sensitivity, make the assay suitable for a test and cull scheme as described in this study.

The ELISA method originally described by Ter Laak and others (1992) and the improved method described by Dercksen and others (2000) has been used as a tool in an established disease eradication and control scheme in the Dutch dairy goat industry. In the latter paper a specificity of 98% +/- 2% and sensitivity of 94% +/- 3% was reported for the improved ELISA when used in goats. A scheme for the eradication of CLA and subsequent accreditation of disease freedom has been available to goat producers in Holland for a number of years (Dercksen and others 1996). In 1996 a total of 46 herds and 11,000 goats had been accredited and trade in stock was then established between members. By 2007 the membership of the scheme had risen to 605 herds, representing approximately 220,000 accredited goats.

A similar health scheme was proposed for the control and eradication of CLA in the smaller Dutch sheep industry using the earlier version of the ELISA test (Schreuder and others 1994), although problems of test sensitivity in some flocks led to the closure of that scheme (Dercksen and others 2000). The test was subsequently modified and the sensitivity in sheep improved by Dercksen and others (2000). However, no further attempts have been made to establish a CLA Health Scheme in what is a relatively small Dutch sheep industry.

Material and methods

(a) Sheep and management

Sheep from six different flocks were clinically examined and blood sampled for testing using an ELISA for CLA at regular intervals over a period of four years. Three of these flocks were located in Scotland, one in England and two in Northern Ireland.

Farm A was a mixed enterprise on around 22 hectares, farming a small herd of pedigree Charolais cattle and a flock of pedigree Suffolk sheep. At the start of the project period

the trial flock was comprised of approximately 50 breeding ewes and was closed in terms of management, apart from the periodic purchase or hiring of rams. A laboratory diagnosis of CLA was made for the first time in February 1997. The source of the CLA infection was considered to be a shearling ram, which had been purchased during the autumn of 1996. After a period in the trial and towards the end of 1999 the flock owner decided to cease active farming. A dispersal sale of the entire Suffolk sheep flock took place shortly thereafter.

Farm B consisted of a suckler cattle herd, a commercial sheep flock and a pedigree Suffolk sheep flock, the latter supplying rams for home use and for sale. A diagnosis of CLA was made within the Suffolk flock for the first time in March 1997. The source of the CLA infection was believed to be a shearling ram that had been leased for a period of six months to flock A. On its return to flock B this sheep and other in-contact rams developed clinical signs of CLA, which were confirmed by cultures. In 2000 stock numbers on the farm were reduced and a flock of approximately 60 breeding Suffolk ewes retained.

Farm C consisted of a pedigree Suffolk flock of approximately 75 breeding ewes and a small livery stable for horses and ponies on approximately 10 hectares. CLA was diagnosed for the first time within this flock in 1999. During the process of establishing the Suffolk flock, breeding sheep had been purchased from a number of sources and it is believed that the infection gained entry to the farm during this process of expansion. Following the initial bacteriological diagnosis of CLA infection, the flock was thereafter managed under essentially closed conditions.

Farm D had a small pedigree Charollais sheep flock of some 20 breeding ewes, as part of a much larger farming enterprise. The purpose of this flock was to produce rams for use in the larger commercial flock, with any additional sheep being sold for breeding. The first diagnosis of CLA was made in 1997 and the infection was believed to have entered the flock through purchased breeding stock within the previous few years.

Farm E consisted of a flock of approximately 60 breeding pedigree Suffolk ewes kept on a smallholding with no other livestock. The first diagnosis of CLA was made in January 1999. The affected sheep had been purchased into the flock in November 1998.

Farm F consisted of a Charollais flock of 25 breeding ewes, which although on the same holding, were managed at separate premises from a commercial flock of some 190 crossbred ewes. The first cases of CLA were diagnosed in July 1999 and involved two shearing rams, both of which had been born on the farm. A further five cases were confirmed in rams between August and November 1999. It was considered that CLA had entered the flock with sheep purchased over the previous two years.

At the commencement of the trial period the respective owners were given specific advice on management changes likely to reduce the spread of CLA infection within their flocks. This included the immediate isolation of suspect clinical cases pending bacteriological confirmation, removing the suspect animals from the rest of the flock and maintaining them in an area where nose-to-nose contact was not possible. Treatment of housing and pens that had held known infected sheep, by means of steam cleaning and application of a disinfectant solution from the Defra approved list at specified concentrations (Anon 2009a) was also recommended. Guidance was given on the application of hygienic measures during procedures likely to cause damage to the skin, such as castration, shearing and tattooing. Finally, any sheep already diagnosed with CLA were required to be either culled, or managed separately for the duration of the trial.

(b) Clinical examinations

Each sheep flock was visited at intervals of approximately six months over the trial period of three to four years. On the holdings that were in the trial during 2001, the application of regulations related to the foot-and-mouth disease epizootic of that year meant that no farm visits could take place between February and November.

All trial sheep were individually identified through the recording of ear marks/tags and examined for clinical signs of CLA. This examination involved the palpation and visual inspection of the superficial lymph nodes of the head, neck and body. Any sheep showing enlarged or abscessed lymph nodes were isolated, and where possible, samples of purulent discharge were taken for bacterial culture. At each visit every sheep over six months of age was clinically examined in this way, after which it was blood sampled.

Blood samples were taken from sheep on farm A on two occasions during 1999. The trial was then discontinued following the sale of the flock. Blood samples were taken from sheep on farm B on seven occasions between May 1999 and June 2003; on farm C on eight separate occasions between July 1999 and May 2003; on farm D on five occasions between July 1999 and August 2003; on farm E on five occasions between February 2000 and July 2003; and on farm F on five occasions between May 2000 and August 2003.

(c) Serology

Sera were examined blind at the Dutch Central Veterinary Institute (ID-DLO), Lelystad, in the Netherlands using the refined ELISA method as described by Dercksen and others (2000). On the basis of the ELISA results samples were designated as negative, inconclusive or positive. When sera gave an inconclusive test reaction in the ELISA, a further Western blot technique was applied (Kamp and others 2001). The Western blot result was taken as positive when at least two bands were clearly visible on the test membrane at 31 kDa and 68 kDa. If the Western blot gave a negative result (no bands), or an unconvincing result (one or two very weak bands) the sample was classified as inconclusive. All sheep classified as seronegative or inconclusive were retained within the flock and re-sampled in the normal manner at the next flock test. All sheep that were considered to be seropositive by ELISA and/or Western blot were recommended to be immediately culled or removed from the flock and managed separately.

(d) Pathology

During the course of the trial any sheep identified as CLA-positive on the basis of clinical examination or serology were either isolated from the rest of the trial flock or culled. A sample of the sheep that were clinically affected and ELISA-positive were subjected to postmortem examination. In addition, where flock management allowed, a number of the ELISA-negative cull sheep were also necropsied. A standard operating procedure was adopted to ensure consistency. Sheep originating from flocks A, B, C and D were examined by the author at the Scottish Agricultural College Veterinary Services, Disease Surveillance Centre, St Boswells. Sheep from flocks E and F were examined by a veterinary colleague at the Agri-Food and Biosciences Institute, Veterinary Sciences Division, Omagh. Each sheep was condition scored and a general external examination of the carcass was undertaken, with particular reference to any enlarged superficial lymph nodes or purulent sinus discharges. A detailed necropsy examination was then undertaken, once again with particular reference to carcass lymph nodes and to the lungs.

Any suspect lesions, and in addition all prescapular, submandibular, parotid, retropharyngeal and a portion of broncho-mediastinal lymph nodes (LN), were cultured and examined as described by Cowan and Steel (1993). Tissues not showing gross lesions of CLA were first dipped in 40% alcohol and flamed to remove any surface contaminating organisms. They were then macerated in nutrient broth using a mechanical stomacher (Wolf Laboratories Ltd, York), prior to plating-out onto blood agar. Plates were then incubated aerobically at 37°C and inspected at 24 and 48 hours. If *C. pseudotuberculosis* was present, visible growth tended to be limited at 24 hours, with colonies reaching a diameter of some 1 to 2 mm at 48 hours. Presumptive identification of recovered bacteria was made on the basis of colony morphology, catalase activity and microscopic examination following standard Gram-staining. A definitive identification of the organism was made using the Analytical Profile Index

(API) Coryne identification system (bioMérieux (UK) Ltd., Basingstoke, Hampshire, UK).

Results

(a) Clinical examinations

Flock A: Prior to the first round of clinical examinations in this flock, eleven sheep had clinical signs consistent with CLA and had been removed from the flock (Figure 5.1). No clinically suspect sheep were detected at the first or second rounds of blood testing and clinical examination in this trial.

Figure 5.1 An adult Suffolk ewe from flock A showing signs of a CLA lesion in the region of the parotid lymph node (picture taken by author).



Flock B: Prior to the first round of clinical examinations in this flock, four sheep had been confirmed as clinically and bacteriologically positive for CLA. A further three sheep had clinical signs consistent with CLA at the first round of testing. No further clinically positive sheep were detected in subsequent rounds of testing.

Flock C: Immediately prior to the first round of clinical examinations in this flock, one sheep had been confirmed as clinically and bacteriologically positive for CLA. At the first round of testing a further thirty sheep had clinical signs consistent with CLA. No further clinically positive sheep were detected in subsequent rounds of testing.

Flock D: Prior to the first round of clinical examinations in this flock, three sheep had been confirmed as clinically and bacteriologically positive for CLA and a further one animal had clinical signs consistent with CLA at the first round of testing. The owner of the flock continued to report visible evidence of disease throughout the trial period.

Flock E: Prior to the first round of clinical examinations in this flock, five sheep had been confirmed as clinically and bacteriologically positive for CLA. There were no further clinical cases of CLA detected throughout the trial period.

Flock F: Prior to the first round of clinical examinations in this flock seven sheep had been confirmed clinically and bacteriologically positive for CLA. Thereafter there were no further clinical cases of CLA detected throughout the trial period.

Contact with the owner of flock B confirmed that the sheep flock remained clear of clinical evidence of CLA until it was sold in 2005. Contact with the owners of flocks C, E and F as recently as July 2009 confirmed that those flocks also remained free of clinical disease.

(b) Serology

The results of the serological examinations in the six trial flocks are summarized in Table 5.1. In all four flocks where testing was completed and management advice on control of infection was followed the serological incidence of infection declined. In flocks B and C seroprevalence reduced to such an extent that at the final two flock tests no seropositive sheep were detected. On the final test, two sheep were seropositive in both flocks E and F. These four animals were subjected to postmortem examination and CLA lesions were not found. The decline in seroprevalence apparent in flock C during the course of the trial is indicated in Figure 5.2. The owners of flock D failed to follow advice given on the removal of seropositive sheep and as a result both seroprevalence and incidence of clinical signs increased during the course of the trial. This steadily rising seroprevalence is indicated in Figure 5.3.

(c) Pathology

A total of 88 postmortem examinations were undertaken on sheep of known serological status from the six trial flocks. Of these, 28 carcasses were found to have gross evidence of abscessation, subsequently confirmed as CLA by bacteriology. CLA lesions were most commonly seen in the lungs (n=13), parotid lymph node (n=10), broncho-mediastinal lymph node (n=8) and prescapular lymph node (n=3) (Table 5.2). In summary, of the 13 sheep with lung abscesses, seven also had lesions in at least one other superficial or visceral lymph node site or within the parenchyma of the liver. Half of the animals examined showed no macroscopic evidence of disease (Table 5.3).

Table 5.1 Results of serial serological examinations in the six trial flocks.

	ELISA test result	Round 1	Round 2	Round 3	Round 4	Round 5	Round 6	Round 7	Round 8	Round 9	Total seropositive sheep
Flock A	Positive	4 (5.3)	1 (1.6)								5
	Negative	71 (94.7)	61 (98.4)								
	Inconclusive	0	0								
	Total sheep	75	62								
Flock B	Positive	17 (10.8)	7 (5.3)	4 (4.2)	1 (0.9)	1 (1.0)	0	0			30
	Negative	135 (85.4)	113 (86.3)	89 (93.7)	99 (88.4)	86 (89.6)	94 (100)	62 (100)			
	Inconclusive	6 (3.8)	11 (8.4)	2 (2.1)	12 (10.7)	9 (9.4)	0	0			
	Total sheep	158	131	95	112	96	94	62			
Flock C	Positive	68 (63.0)	12 (34.3)	5 (8.5)	18 (37.5)	22 (44.0)	19 (26.4)	15 (21.4)	0	0	159
	Negative	30 (27.8)	23 (65.7)	54 (91.5)	29 (60.4)	27 (54.0)	52 (72.2)	55 (78.6)	94 (98.9)	41 (100)	
	Inconclusive	10 (9.3)	0	0	1 (2.1)	1 (2.0)	1 (1.4)	0	1 (1.1)	0	
	Total sheep	108	35	59	48	50	72	70	95	41	
Flock D	Positive	17 (28.8)	9 (17.6)	16 (35.6)	14 (66.7)						56
	Negative	39 (66.1)	42 (82.4)	23 (51.1)	4 (19.0)						
	Inconclusive	3 (5.1)	0	6 (13.3)	3 (14.3)						
	Total sheep	59	51	45	21						
Flock E	Positive	5 (7.8)	1 (1.7)	2 (2.9)	0	2 (2.9)*					10
	Negative	59 (92.2)	51 (89.5)	63 (92.6)	80 (98.8)	62 (91.2)					
	Inconclusive	0	5 (8.8)	3 (4.4)	1 (1.2)	4 (5.9)					
	Total sheep	64	57	68	81	68					
Flock F	Positive	0	2 (9.1)	0	0	2 (9.1)*					4
	Negative	21 (91.3)	19 (86.4)	23 (100)	22 (100)	19 (86.4)					
	Inconclusive	2 (8.7)	1 (4.5)	0	0	1 (4.5)					
	Total sheep	23	22	23	22	22					

() Figures in parenthesis represent percentage of samples examined.
 * No lesions detected on examination of animals *post mortem*.

Table 5.2 Site of caseous lymphadenitis (CLA) lesions in 28 sheep.

Site of culture +ve CLA lesions	Number (%) of sheep with CLA lesions (N=28)
Lungs	13 (46%)
Parotid lymph node	10 (36%)
Broncho-mediastinal lymph node	8 (29%)
Prescapular lymph node	3 (11%)
Submandibular lymph node	2 (7%)
Liver	2 (7%)
Prefemoral lymph node	1 (4%)

Table 5.3 Distribution of caseous lymphadenitis (CLA) lesions in 28 sheep.

Distribution of culture +ve CLA lesions	Number of sheep (%)
Internal CLA lesions only (lungs and/or visceral lymph nodes)	14 (50%)
CLA lesions in superficial lymph nodes only	8 (29%)
Both internal and superficial CLA lesions present	6 (11%)

Figure 5.2 Seroprevalence of CLA in flock C during course of the eradication trial.

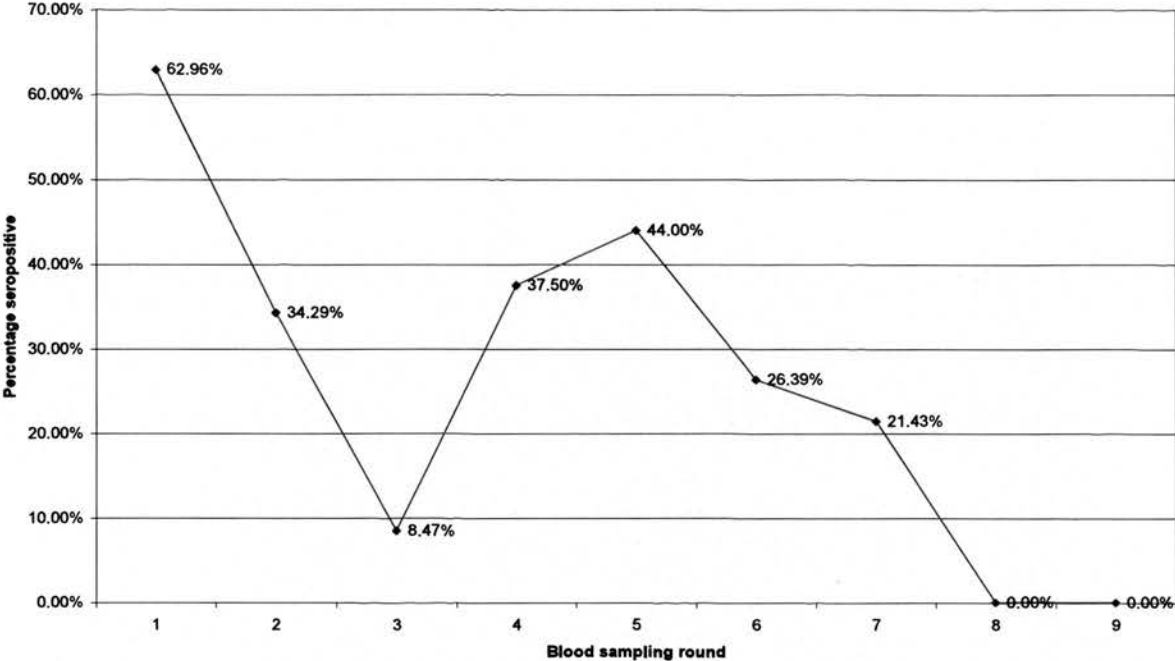
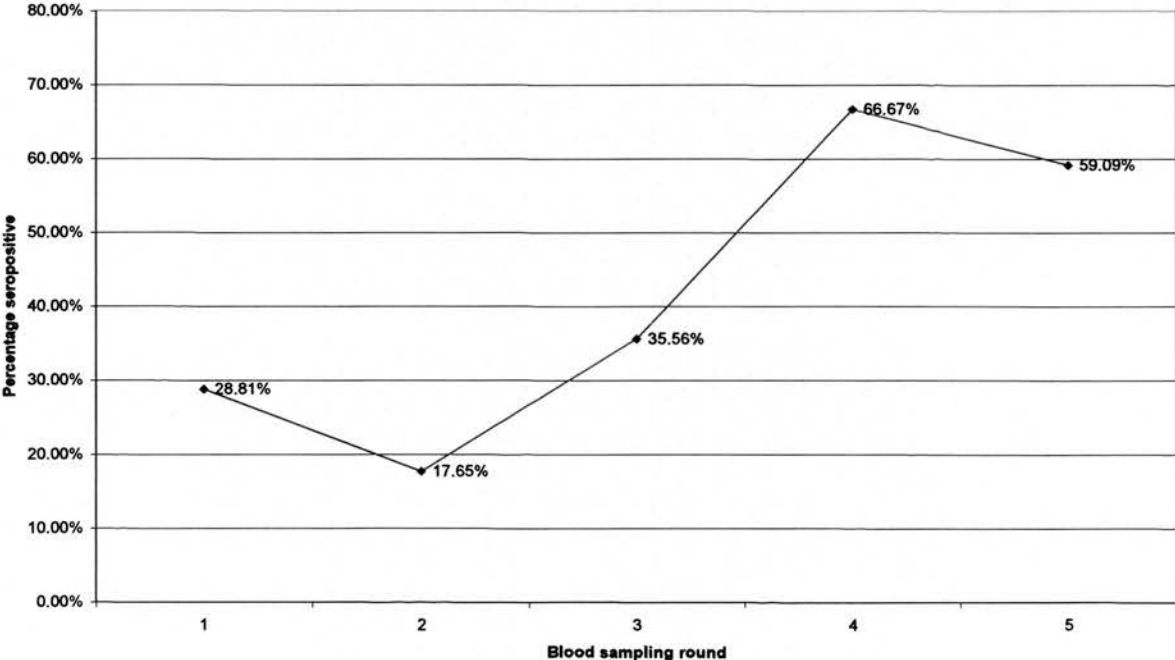


Figure 5.3 Seroprevalence of CLA in flock D during course of the eradication trial.



Discussion

Since the arrival of *C. pseudotuberculosis* in the UK the prevalence of CLA within the sheep industry has risen inexorably. The proprietary vaccines used so widely to control CLA in other parts of the world have not been licensed for use in many European countries including the UK. Farmers concerned about the spread of infection between and within flocks have therefore had to rely on clinical examination alone to identify affected sheep.

Whilst serological testing has been employed as a research tool in a number of other countries (Chikamatsu and others 1989; Kuria and Holstad 1989a; Ellis and others 1990; Sutherland and others 1992), the use of blood tests to control and eradicate disease has not been practised widely. The development of an ELISA method for detecting antibody to the PLD exotoxin of *C. pseudotuberculosis* (Ter Laak and others 1992) led to the establishment of a CLA health scheme within the Dutch dairy goat sector (Dercksen and others 1996). This scheme established a means whereby the owners of infected goat herds could eradicate CLA, with the ultimate aim of becoming an accredited CLA-free herd.

In this study it has been established that the regular use of an ELISA to test and remove positive sheep, in association with clinical inspections and changes in farm management, can greatly reduce the incidence of CLA in affected UK flocks. Additionally, the finding that 50% of sheep with gross lesions showed only the internal or visceral form of CLA, was somewhat higher than in previous reports from the UK, where between a quarter and a third of sheep were affected in this way (Malone and others 2006; Baird and Fontaine 2007). Approximately one half of the visceral lesions identified in this study were located within the lung parenchyma. Sheep affected with such pulmonary lesions have been identified by several authors as the principal source of infection to other sheep (Ellis and others 1987; Pepin and others 1994a). The first mathematical

model of *C. pseudotuberculosis* infection indicated that while transmission from superficial lesions may predominate during the early spread of infection within a naive population, transmission from respiratory abscesses dominates the endemic phase of disease (O'Reilly and others 2008). This indicates that CLA management based purely on the removal of visibly affected sheep is unlikely to be successful. The possibility that serology might be employed to identify such sheep therefore offers the opportunity to exert enhanced disease control.

The most significant effects of such a strategy were seen in trial flock C, where at the commencement of the study seroprevalence was greater than 60%, and approximately 40% of all adult sheep showed clinical signs of disease. Although seven rounds of testing over a period of three years were required to remove all seropositive sheep from this flock, the two final tests, which took place six months apart, failed to identify any antibody positive sheep. No further clinical cases of CLA were detected by the owner during the following five years. Although clinical inspection cannot detect the minority of animals with the internal form of the disease alone, the absence of external signs of CLA for this length of time in the view of the author constitutes effective eradication of disease. In future, postmortem examination of fallen stock or abattoir examination of cull sheep may be suitable methods of disease monitoring in flocks that have adopted CLA eradication by regular ELISA testing.

This study indicates that effective control over CLA may be achievable in flocks with the highest prevalence, through the use of serology allied to management changes. However, it also makes clear that a reduction in disease prevalence is not possible in situations where owners are insufficiently motivated to make the required changes in management, or are unwilling to remove clinically or serologically positive sheep. This was indicated most clearly in trial flock D, where advice on control of infection and culling was not followed, and as a consequence both the clinical and serological incidence of CLA increased during the course of the study.

This trial was fully funded, such that flock owners were not required to pay for any testing or veterinary visits and received payment for those animals that were culled and subjected to necropsy examination. If such a test and cull programme was to operate commercially, this would not be the case. As a consequence, it would seem likely that the owners of CLA-affected flocks of high value and small size would be most likely to consider such a scheme. For the owners of larger flocks, where the costs of testing, culling and sourcing replacements would be correspondingly greater, the chances of such a scheme proving attractive would be much less.

Chapter 6: CONTROL OF CASEOUS LYMPHADENITIS IN A LARGE UPLAND SHEEP FLOCK BASED ON CLINICAL EXAMINATION AND REGULAR ELISA TESTING.

Summary

The application of a test and cull policy to control CLA in a commercial hill flock in northeast Scotland is described. A commercially available ELISA test kit (ELITEST CLA ELISA, Hyphen, France) for the detection of antibody to PLD was employed on blood samples taken from the main flock at approximately three-monthly intervals between November 2007 and August 2009. A Western blot assay was used on occasions when ELISA test results were considered inconclusive. Following each round of testing all animals identified as seropositive were removed from the flock. Similarly, any sheep diagnosed as CLA-positive through clinical examination and bacteriology, were also removed from the flock. In addition, a range of general hygiene and management measures aimed at reducing the likelihood of disease transmission were also encouraged on the holding.

Initial testing in November 2007 indicated a seroprevalence of CLA within the flock of 10% (n=1010). By August 2009 the application of the test and cull protocol described, the flock seroprevalence was reduced to 0.4% (n=538). No cases of CLA have been identified within the flock on the basis of clinical examination from June 2008 to date (November 2010); however in May 2009 CLA was confirmed at necropsy examination in a ewe that had previously tested positive serologically. The potential role of serological testing as a tool in the eradication of CLA from large flocks under extensive hill conditions is discussed.

Introduction

As described in Chapters 3 and 5, the increase in CLA prevalence recorded in the UK since the 1990s has occurred principally within the terminal sire breeds, in relatively intensive lowland farming systems (Baird and others 2004). However by the mid-2000s, reports began to emerge of CLA being detected in extensively managed hill flocks. Predominantly this was in the North Country Cheviot and Scottish blackface breeds (Anon 2004, 2007c), with infection commonly identified for the first time in rams. Investigation of these outbreaks identified as a common risk factor, the practice of overwintering rams from hill flocks on low ground holdings on the east coast of Scotland. Here rams from a number of different flocks are managed together in very large groups. These ram groups are composed of animals of varied breeds and from many different farms. It seems likely that contact between the rams during the winter months enabled the transmission of CLA to rams of the hill breed, which then introduced the infection to their holding of origin.

This trial was conducted in a Scottish blackface flock in the north of Scotland. Caseous lymphadenitis was first diagnosed in the flock in August 2003 when *C. pseudotuberculosis* was isolated from lesions identified on a Texel and a Charollais ram, both of which were three-years-old and purchased for breeding purposes in 2002. The case history indicated that during the previous year, the same Texel ram had suffered an abscess that had been lanced and flushed, and had appeared to heal. Following the diagnosis of CLA in 2003, both affected animals were culled.

Between August 2003 and December 2006, swabs were submitted from superficial purulent lesions affecting rams on a total of 12 occasions. In three cases CLA infection was confirmed by bacteriology and the affected animals were culled. This practice appeared to control the spread of infection, and when the 17 rams within the stud in August 2006 were tested, no CLA ELISA positive animals were detected. However, around shearing time in the summer of 2007, a number of ewes and gimmers were identified with enlarged or abscessed superficial lymph nodes. At the time, the visiting

local veterinary practitioner estimated a 5% prevalence of such lesions, mostly affecting the nodes of the head and neck. Samples were taken for bacteriology from 18 animals and a growth of *C. pseudotuberculosis* subsequently confirmed in nine cases. These nine and another 31 animals with enlarged lymph nodes in the head and neck area were culled at that time.

Chapter 5 describes the use of an antibody ELISA as part of a test and cull programme in six small CLA-affected lowland sheep flocks. For two of those flocks it was concluded that complete disease eradication had been achieved, while in another two, the incidence of clinical CLA was reduced to zero, although a small number of seropositive animals could still be detected. In this trial similar principles of disease eradication were applied to a much larger flock in a more extensive hill setting. While the logistics of such a programme were more demanding, the basic model remained the same: the testing of the whole flock at regular intervals, allied with increased farmer vigilance and application of management changes aimed at reducing the transmission of infection.

Materials and methods

(a) Sheep and management

The trial flock was located on a holding in Ross-shire, Scotland. No other livestock were kept on this holding, which covered approximately 500 ha at an elevation of between 100 to 500 metres, with the majority of grazing on rough hill ground with a limited amount of improved pasture. In November 2007 the flock consisted of some 670 Scottish blackface ewes, 180 ewe hoggs and 30 mule ewes. The ram stud was composed of 15 Scottish blackface rams, seven Bluefaced Leicester rams and a single Suffolk ram.

Established practice in the flock was for all Scottish blackface female replacements to be homebred, while the majority of female mule progeny were sold as breeding animals. All male blackface and mule lambs were sold at light slaughter weights or as stores. In

addition, the small group of mule ewes were bred to the Suffolk ram, with all progeny sold fat. Prior to the commencement of the trial all replacement breeding rams were sourced locally. No serological screening of these purchased male animals was performed, nor was any period of quarantine observed prior to their introduction to the stud.

Female lambs were away-wintered on a lowland farm where there was no contact with other sheep. The majority of the flock was grazed on hill pastures or on in-bye grassland throughout the year. Only twin-bearing ewes were housed for a short period around lambing. Grazing animals were supplemented with hay or silage provided in round feeders as necessary. Shearing each year was carried out by the farmer with local help.

At the commencement of the trial period the owner of the flock was given specific advice on management changes designed to reduce the likelihood of CLA transmission. This included the immediate isolation of suspect clinical cases pending bacteriological confirmation, removing the suspect animals from the rest of the flock to an area where nose-to-nose contact was not possible. Treatment of housing and pens that had held known infected sheep, by means of steam cleaning and application of a disinfectant solution from the Defra approved list at specified dilutions (Anon 2009a) was also recommended. During shearing, any animals known to be seropositive or clinically affected by CLA were sheared last. If any sheep was discovered to have abscesses at shearing, the blades used were disinfected before being used on the next animal.

Ectoparasite control within the breeding stock was based upon the use of injectable drugs and pour-on treatments during the first year of the trial. Thereafter dipping of ewe lambs for ectoparasite control was allowed, provided that sheep from seronegative groups were treated prior to sheep from the seropositive groups. Ectoparasite control remained by pour-on treatment and injectable drugs in the adult breeding flock. Similarly whenever the flock was handled for worming, shearing, scanning or any other procedure, the seronegative group was handled before any retained seropositive animals.

Following such handling the holding areas and the race were to be thoroughly cleaned and disinfected before the next use.

Guidance was given on the application of hygienic measures during procedures likely to cause damage to the skin, such as castration, shearing and tattooing. No hypodermic needles used in vaccination or parasite treatments were shared between seropositive and seronegative groups of animals. Finally, any sheep already diagnosed with CLA was either culled, or managed separately for the duration of the trial.

All breeding rams introduced into the flock during the trial period were either certified under the CLA Monitoring Scheme, or appropriately quarantined for at least two months, with clinical examination and ELISA testing at the beginning and end of the quarantine period. The advice to the owner was that female breeding replacements should only be retained from those dams with no evidence of visible lesions or serological history of CLA infection. However, in the first year of the trial when insufficient ewe lambs were available from seronegative dams, 30 female progeny born to ewes in the seropositive group were retained. As a precaution, these 30 ewe lambs were run as a separate group until they had passed three consecutive blood tests, at which time they were then added to the main breeding flock.

(b) Clinical examinations

The trial flock was visited on eight occasions at intervals between November 2007 and August 2009. On each occasion every sheep over six months of age was individually identified through the recording of ear tags, subjected to a brief clinical examination and blood sampled. The physical examination involved the palpation and visual inspection of the superficial lymph nodes; to include the regions of the parotid, submandibular, retropharyngeal, prescapular and prefemoral nodes. The identity of any sheep showing enlarged or abscessed lymph nodes was noted. Where a sample of discharge could be collected the animal in question was separated from the flock pending the results of bacteriology. In these cases bacterial cultures were performed under standard aerobic,

anaerobic and 10% CO₂ conditions at SAC Veterinary Services Centres in Inverness or Perth. If *C. pseudotuberculosis* was confirmed on culture the affected animal was immediately culled or removed from the main flock and managed separately.

In addition to the visits by a veterinary surgeon, the owner of the flock was also asked to examine sheep for clinical signs of CLA at every handling, and especially at shearing. Any discharging skin lesions identified at these times were to be sampled and the swabs sent for bacteriological examination.

(c) Serology

Blood samples were taken from animals in the main flock on eight occasions between November 2007 and August 2009. These samples were submitted to SAC Veterinary Services Inverness, where they were assayed for the presence of anti-PLD IgG by ELISA using the ELITEST CLA (HYPHEN BioMed, France).

The ELISA test was performed according to the manufacturer's instructions, as follows: microplate strips supplied in the kit were coated with highly purified recombinant PLD (rPLD) constituting the solid phase antigen. The test sample was incubated in the well and specific antibodies to rPLD, if present in the sample, bound to the solid phase immobilized antigen. A mouse monoclonal anti-goat/sheep IgG labelled with horseradish peroxidase (HRP) was then added. When IgG antibodies were present, these reacted with the coated antigen and were revealed by the HRP-labelled second antibody. Subsequent incubation with tetramethylbenzidine peroxidase (TMB/H₂O₂) substrate produced a blue colour in the test well, which turned to yellow when the reaction was stopped by the addition of 100 µl of 2 M sulphuric acid. If the sample contained no antibodies, the HRP-labelled second antibody did not bind and no colour developed. The absorbance of each sample at light wavelength 450 nm was then determined using an ELISA plate reader.

On the basis of the ELISA results, serum samples were designated as negative or positive. When sera gave a low positive test reaction in the ELISA, a further Western blot technique (Burnette 1981) employing recombinant PLD antigen supplied by the ELISA kit manufacturers was carried out on the sample at SAC VS Inverness. The Western blot result was taken as positive when at least two bands were clearly visible on the test membrane at 31 kDa and 68 kDa. If the Western blot gave a negative result (no bands), or an unconvincing result (one or two very weak bands) the sample was classified as inconclusive.

All sheep classified as seronegative were retained within the flock and re-sampled in the normal manner at the next flock test. All sheep that were considered to be seropositive by ELISA and/or Western blot were immediately culled or removed from the flock and managed separately. Sheep that had shown an inconclusive result were isolated for four weeks before being blood sampled and re-tested in the ELISA.

At the first farm visit in November 2007 all breeding sheep present on the farm were blood sampled and tested by CLA ELISA. This included a group of older ewes that had already been selected for culling on the basis of age, body condition or history of abscesses. This group was included in the flock test in order to achieve a more complete record of CLA seroprevalence at the commencement of the trial. All ewe lambs born in spring 2007 were also blood sampled at this first visit in order to assess the extent of the spread of infection into the lamb group.

(d) Pathology

After the initial flock visit in November 2007, and after all subsequent tests, any sheep confirmed as CLA positive by bacteriology or deemed positive in the ELISA and/or Western blot assays was removed from the main flock. On most occasions those animals with superficial CLA lesions were immediately culled; whilst those sheep that were simply seropositive but with no visible abscesses, were kept separate until removal. At intervals during the trial a proportion of the animals from this latter category were

subjected to full necropsy examination, both to monitor test specificity and to assess the extent of internal CLA lesions. A standard operating procedure was adopted in all postmortem examinations to ensure consistency (see Chapter 5). These examinations were carried out at SAC VS Inverness by the author.

Results

(a) Clinical examinations

In the period between the commencement of the trial in November 2007 to the end of May 2010, swab samples taken from superficial abscesses were submitted on 22 occasions, with a total of 27 sheep sampled for culture. On no occasion was *C. pseudotuberculosis* identified on subsequent bacterial culture. In more than 50% of cases growths of either *Arcanobacterium pyogenes* or *Actinobacillus lignieresii* were identified, or cultures proved sterile after 48 hours incubation. Other organisms isolated on culture from individual animals were identified as *Actinomyces hyovaginalis*, *Staphylococcus epidermidis*, *Bibersteinia trehalosi*, *Staphylococcus aureus* and *Bacillus licheniformis*.

(b) Serology

Western blot analysis was carried out on all ELISA positive sera after the first round of testing. As the trial progressed and seroprevalence by ELISA test declined, all positive samples were again tested by Western blot. These results are shown in Table 6.1.

Table 6.1 Serial serological examinations in trial flock.

Date of testing	Animals sampled	No. of animals seropositive by ELISA	Seropositive by ELISA (%)	Positive Western blot
Nov 2007	1,010	101	10.0	94/101
Feb 2008	781	16	2.0	N/A
May 2008	711	6	0.8	N/A
Aug 2008	515	2	0.4	N/A
Nov 2008	707	12	1.7	10/12
Feb 2009	693	4	0.6	4/4
May 2009	664	8	1.2	4/8
Aug 2009	538	2	0.4	2/2

(N/A - not applicable, no Western blots performed on those occasions)

In November 2007 the overall seroprevalence in the flock was 10%. None of the rams within the flock were seropositive at the time of testing (n=23), whilst seroprevalence was greatest in the group of cull ewes at 27.3% (n=205). This group contained a large proportion of animals with a clinical history of suspicious superficial lesions. In the group of ewe lambs 1.8% of animals were seropositive (n=228), while 7.4% of the remaining ewes and gimmers (n=554) tested positive for PLD antibody. Following the flock blood test carried out in August 2009 two animals were found to be positive for antibody by ELISA and Western blot, indicating a flock seroprevalence of 0.4% (n=538).

(c) Pathology

A total of eleven necropsy examinations were undertaken on trial sheep. In November 2007, five animals that had previously tested positive by ELISA were examined. In four cases purulent lesions were noted in the superficial or thoracic lymph nodes or the lungs, from which *C. pseudotuberculosis* was subsequently isolated. In one case no purulent lesions were detected and routine cultures from sampled lymph nodes were negative.

In August 2009, five of the eight animals that had tested ELISA positive in May 2009 were subjected to a necropsy examination, the other three animals having been sold for slaughter shortly after the test. On this occasion the delay of three months between

blood testing and necropsy examination was to allow these ewes to rear their lambs. Following euthanasia, gross inspection of one carcass identified slight enlargement of the right prefemoral lymph node and when incised the node was found to contain a small quantity of purulent material. On subsequent culture of the contents of this lesion a growth of *C. pseudotuberculosis* was identified. No lesions were recorded on necropsy examination of the remaining four carcasses and routine lymph node cultures produced no significant findings.

In September 2009 one of the two ELISA positive/Western blot positive animals tested in August 2009 was examined at necropsy examination, the other having been sold for slaughter by the owner. No lesions were recorded on examination of this animal and routine lymph node cultures produced no significant findings.

Discussion

This study confirms that the programme of CLA control, described for lowland pedigree sheep flocks in Chapter 5 – namely a test and cull policy employing a PLD antibody ELISA assay, allied to an increased farmer vigilance and certain management changes - may be successfully applied to reduce the prevalence of the disease in a large commercial hill flock. In this case, *C. pseudotuberculosis* infection was well established within the flock before the commencement of the trial. In the four years following the first confirmed diagnosis of CLA within the ram stud, the infection appeared to have been controlled in that group through the immediate culling of affected animals. However, the discovery of clinical signs of CLA in an estimated 5% of ewes between June and July 2007, and thereafter the detection of 10% seroprevalence in November 2007, confirmed active infection with the lambing flock. Through application of the test and cull programme this seroprevalence was reduced to a very low level and evidence of clinical disease eliminated for a period of 31 months (at time of writing in July 2010). At the end of this trial the author would have welcomed an opportunity for one further round of testing, in the hope of establishing an entirely seronegative status

within the flock. Unfortunately the limitations of funding meant that this was not possible.

Although there was no history of carcase rejections, weight loss or other systemic effects of CLA, the sudden rise in disease incidence recorded within the female stock during 2007 led to great concern on the part of the owner. For an enterprise based on the sale of quality female breeding replacements, it was felt that the reputation of the business would be put at risk were CLA-affected stock to be marketed. The flock owner was therefore enthusiastic and highly motivated throughout the trial. As a consequence, he was willing to accept the additional efforts required in gathering the flock for testing and in applying the management measures aimed at reducing disease transmission. In addition, because the flock was undergoing a period of managed reduction, the owner could accept the premature loss of the 151 animals that were to test seropositive during the course of the trial, and the further 43 animals that were either diagnosed with CLA by bacteriology before the trial commenced or were removed on the basis of suspicious lesions.

Although the owner saw CLA control in this flock as being an economic imperative, without financial support of the veterinary visits for blood-sampling and all serological testing, an eradication programme of this type would not have been possible. During the trial period, the equivalent commercial cost of the CLA ELISA test was between £4.00 and £4.50 per sample. Given that the trial involved eight separate veterinary visits for blood-sampling, and that a total of 5,619 samples were tested, the likely costs for this part of the trial would have been in the region of £30,000, had it been under commercial conditions.

As discussed in Chapter 5, the definition of complete disease eradication is important in assessing this trial. The absence of any confirmed cases of superficial CLA for a period of 31 months is consistent with effective eradication of this form of the disease. However, the trial also confirms how difficult it is to achieve complete eradication of *C.*

pseudotuberculosis infection in a large flock under extensive conditions. The detection of two seropositive animals after eight rounds of testing, and the discovery of one animal with the visceral form of infection during a necropsy examination in August 2009, confirms this problem.

In the accreditation schemes for maedi-visna and *Chlamydomphila abortus* (enzootic abortion of ewes, EAE) currently operated in the UK, an absence of any serological evidence of infection is a requirement of full membership (PSGHS 2008, 2009). The nature of caseous lymphadenitis infections in sheep and the relatively modest sensitivity of the ELISA test would make this a difficult objective to achieve in any large flock suffering a significant level of infection.

In conclusion, it would seem likely that the owners of CLA-affected flocks of high value and small size would be most likely to consider such a test and cull programme. For the owners of larger flocks, the costs of testing, culling and sourcing replacements are correspondingly greater. Although this trial has shown that effective control can be applied in such situations, on the basis of a cost-benefit analysis, such a programme is unlikely to prove attractive for the majority of larger producers.

Chapter 7: VACCINATION TO PROTECT SHEEP AGAINST A VIRULENT UNITED KINGDOM STRAIN OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS*.

Summary

Using a virulent United Kingdom *C. pseudotuberculosis* isolate, an ovine experimental model of caseous lymphadenitis was developed, in which the manifestation of disease was equivalent to the naturally-observed infection in this country. The ability of several experimental vaccines to protect against bacterial challenge was then determined. Sheep were immunized with a recombinant PLD derived from the virulent UK isolate, a formalin-killed bacterin of the same strain, or a bacterin supplemented with the recombinant PLD. Following experimental homologous challenge, the PLD and bacterin vaccines were observed to confer statistically significant protection against infection, and restricted dissemination of challenge bacteria beyond the inoculation site in the majority of animals. More importantly, the combined vaccine succeeded in providing absolute protection against infection, whereby challenge bacteria were eradicated from all vaccinates. In addition to the experimental vaccines, a commercially available CLA vaccine, currently unlicensed for use in the European Union, was assessed for its capacity to protect against heterologous challenge. The proprietary vaccine conferred significant protection, although the dissemination of infection beyond the inoculation site was not restricted as it had been with the other vaccines. However, no animals vaccinated with this proprietary product manifested infection within the lungs; thus eliminating a potentially important route of disease transmission. The results of this study provide information pertinent to the development of an effective caseous lymphadenitis vaccination strategy in the UK.

Introduction

The potential to vaccinate sheep against infection with *C. pseudotuberculosis* has been the focus of research attention for many years. A prepared vaccine against CLA was said to be used to protect flocks in France as early as the 1920s (Howarth and Young 1926). Bacterin vaccines, or those comprising cellular components, have been shown to offer a degree of protection against experimental infection (Cameron and others 1972; Brogden and others 1984a, 1985; Brogden and others 1990). In addition, they have also shown some success when used to vaccinate against naturally acquired infection in sheep and goats (Menzies and others 1991; Brogden and others 1996).

Some of the earliest investigations into vaccination against *C. pseudotuberculosis* infection reported the capacity of the potent *C. pseudotuberculosis* exotoxin to induce protective immunity in guinea pigs (Petrie and McClean 1934). Despite these encouraging results, the consensus view at the time was that the chronic nature of *C. pseudotuberculosis* infections in sheep, compared to the relatively acute manifestation in guinea pigs, meant that exotoxin vaccines were unsuitable for use against ovine CLA (Carne and others 1956; Cameron and Smit 1970). Later work revealed that the *C. pseudotuberculosis* exotoxin, now characterised as PLD (Cameron and Smit 1970), did in fact offer protection against infection in sheep (Jolly 1965). Subsequent studies employed formalin-inactivated toxoid vaccines derived from PLD-rich *C. pseudotuberculosis* culture supernatants, and established that this conferred varying levels of protective immunity in both sheep and goats (Jolly 1965; Nairn and others 1977; Burrell 1978b; Eggleton and others 1991a).

Currently there is no commercially available CLA vaccine licensed for use in the UK. Permission may be sought by veterinary practitioners from the UK Veterinary Medicines Directorate (VMD) for the importation of proprietary vaccines under a Special Treatment Certificate or for the production of autogenous vaccine under the Autogenous Vaccine Authorisation arrangements. The latter are adjuvanted bacterins derived from *C. pseudotuberculosis* isolates from flock outbreaks and are intended only for the

immunisation of animals on the holding where the outbreak occurred. However, the protective capacity of this type of relatively crude preparation has been purely anecdotal. Similarly no assessment of the proprietary vaccines available in other countries has yet taken place in the UK.

In this study the capacity of a *C. pseudotuberculosis* bacterin to protect sheep against homologous challenge with a virulent UK isolate of *C. pseudotuberculosis* was assessed. In addition, the capacity of a recombinant derivative of PLD and of a recombinant PLD-supplemented *C. pseudotuberculosis* bacterin to protect against equivalent challenge was evaluated. Finally, the capacity of an Australian commercial vaccine preparation to protect against heterologous challenge with a UK *C. pseudotuberculosis* strain was also assessed.

Materials and methods

(a) Media and culture conditions

C. pseudotuberculosis was propagated on blood agar (BA) plates derived from Blood Agar Base (Oxoid) and 5% (v/v) sheep blood, or in Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, Hampshire, UK), at 37°C (mixed at 200 rpm for liquid cultures) for up to 48 hours. Unless otherwise specified, *Escherichia coli* strains were routinely cultured at 37°C (mixed at 200 rpm for liquid cultures) in Luria Bertani medium (LB; Oxoid) containing ampicillin (100 µg/ml), or carbenicillin (50 µg/ml) and 10 mM glucose as required. This widely used rich medium broth is popular with bacteriologists because it permits fast growth and good growth yields for many bacterial species (Sezonov and others 2007).

(b) Molecular biological techniques

Unless otherwise specified, all DNA manipulations were performed according to standard techniques (Sambrook and Russell 2001). Restriction endonucleases and other modifying enzymes were purchased from Promega (Southampton, UK) or Roche (East

Sussex, UK) and used according to the manufacturers' supplied instructions. Small-scale extraction and purification of plasmid DNA from *E. coli* was performed using the QIAquick Spin Miniprep Kit (QIAGEN, Crawley, West Sussex, UK), and genomic DNA was extracted from *C. pseudotuberculosis* using the NucleoSpin Tissue Kit (BD Biosciences (Clontech), Erembodegen, Belgium). PCR-amplified DNA fragments were purified using the Strataprep PCR Purification Kit (Stratagene, La Jolla, CA, USA), and cloned into pPCR-SCRIPT using the PCR-SCRIPT Cloning Kit (Stratagene). For cloning, restriction endonuclease-digested DNA fragments were electrophoresed through 1.0% (w/v) agarose gels and fragments of the desired size were purified using the QIAquick Gel Extraction Kit (QIAGEN). Custom PCR and sequencing primers were synthesised to order by Sigma-Genosys (Sigma-Genosys Ltd., Haverhill, Suffolk, UK), and are presented in Table 7.1. Automated DNA sequencing was performed using a MegaBACE sequencing apparatus (Amersham Biosciences UK Ltd., Buckinghamshire, UK) by the Functional Genomics Unit of the Moredun Research Institute, Pentlands Science Park, Penicuik, Scotland.

(c) Cloning of the *C. pseudotuberculosis* *pld* gene

The gene encoding for the PLD exotoxin (designated *pld*) of the virulent UK *C. pseudotuberculosis* isolate, 3/99-5 (Connor and others 2000) was amplified by PCR. The published *pld* sequence (GenBank accession #L16586) was used to design the primers *pld*#01b and *pld*#02, and to facilitate cloning, primers were designed to contain restriction endonuclease cleavage sites. The amplified fragment, corresponding to the truncated *pld* gene lacking the first 72 base pairs (encoding the 24 amino acid (aa) secretion signal sequence), was cloned into pPCR-SCRIPT and the resulting construct was designated pMMF003. To allow expression of the truncated 3/99-5 *pld* gene, pMMF003 was digested using the restriction enzymes *Nde*I and *Bam*HI, and the 859 base pair fragment was ligated into pET-15b. Following transformation of *E. coli* XL10-Gold Kan (Stratagene), the desired construct was identified and designated pMMF004. DNA sequencing of both leading and complementary strands was

performed using the primers pET(fwd) and pET(rev), to confirm the identity of the cloned fragment.

(d) Expression and purification of recombinant PLD (rPLD)

To allow expression of the cloned *pld* gene, pMMF004 was used to transform *E. coli* BL21(DE3). Bacterial cultures were propagated in LB containing carbenicillin and glucose and protein expression was induced in the exponential growth phase (OD_{600nm} of ≈ 0.6) by the addition of IPTG to 1 mM final concentration. Induced cultures were incubated for one hour prior to the addition of rifampicin to 150 $\mu\text{g/ml}$ final concentration. Incubation was continued for a further three hours, and then cells were harvested by centrifugation at $6,000 \times g$ for 15 minutes at 4°C . Culture supernatants were discarded and cells were resuspended in BugBuster Protein Extraction Reagent (Merck Biosciences (Novagen), Nottingham, UK) to approximately 5 ml per gram of wet cell pellet. Complete Protease Inhibitor Cocktail (Roche) was added ($1\times$ final concentration) to inhibit proteolytic degradation, then Benzonase enzyme (Novagen) was added to 1 $\mu\text{g/ml}$ final concentration, and incubation was performed with gentle rotation on a shaking platform for 40 minutes at room temperature. During this incubation, cells were lysed and viscosity due to nucleic acids was reduced. Subsequently, centrifugation was performed at $16,000 \times g$ for 30 minutes at 4°C to pellet cell debris, and then cleared supernatants were supplemented with imidazole to a final concentration of 10 mM.

rPLD was purified by immobilised metal-ion affinity chromatography. Twelve ml EconoPak columns (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) were loaded with 2 ml (bed volume) of Ni-CAM HC resin (Sigma-Aldrich Company Ltd., Irvine, UK), pre-equilibrated with lysis buffer (Column Buffer (CB) [50 mM Tris-HCl, pH 8.0 and 300 mM NaCl] supplemented with 10 mM imidazole). To each column was added 5 ml of cell lysate (originating from approximately 500 ml of culture), and the volume was supplemented with a further 5 ml of lysis buffer. Columns were sealed and incubated for 16 hours at 4°C on a tube rotator. Subsequently, columns were drained by gravity

flow and washed with 25 × 2 ml of wash buffer (CB supplemented with 20 mM imidazole). Finally, column-bound protein was eluted in 5 × 2 ml of elution buffer (CB supplemented with 250 mM imidazole). Eluted proteins were concentrated and buffer exchanged in 10 mM PBS (pH 7.2), supplemented with 0.1% SDS and 1× Complete protease inhibitors, through 10 K M_r Amicon Ultra centrifugal filters (Millipore, Watford, UK) at 3,080 × g and 4°C. The final concentration of purified rPLD was determined using the DC Protein Assay (Bio-Rad).

(e) Preparation of challenge inoculum

C. pseudotuberculosis 3/99-5 (a bacterium originally isolated from a CLA affected sheep in a Scottish Borders flock) was passaged three times on BA plates, and then bacterial growth was collected using sterile, cotton-tipped swabs. Bacteria were resuspended in PBS, in polystyrene Universal tubes, by gently rolling swab tips against the tube sides. Dilutions of the primary suspension were prepared to give the required concentration (cells/ml) for subsequent challenge. This was estimated by use of a Densimat (bioMérieux, Basingstoke, UK), and then quantified accurately using a Thoma counting chamber.

(f) Preparation of formalin-killed C. pseudotuberculosis

For vaccination, *C. pseudotuberculosis* was cultured on BA plates, as for Section 2.5. Approximately 10¹¹ cfu were suspended in 20 ml of PBS in a Universal tube and supplemented with 40% formalin to a final concentration of 0.2% (v/v). Cells were incubated overnight at 4°C, and then quantified using a Thoma counting chamber. Subsequently, cells were pelleted by centrifugation at 3,080 × g for 15 minutes at 4°C, and resuspended in distilled water to a final concentration of 1.25×10¹⁰ cells/ml. To confirm complete killing, 100 µl aliquots of the bacterial suspension were plated onto blood agar plates, then incubated at 37°C and monitored daily for five days.

(g) Experimental challenge and vaccination

For all challenge/vaccination experiments, one-year-old male Suffolk-cross sheep were used. These animals were sourced from the Institute's own closed sheep flock, which was known to be CLA-free and each was tested by CLA ELISA to establish a seronegative status prior to the start of the trial.

Prior to undertaking the vaccination study, a dose titration experiment was performed to determine the minimum cells/ml required to induce experimental infection manifesting clinical signs and pathology similar to naturally observed CLA. Groups of two animals were inoculated with 10^2 , 10^3 , 10^4 or 10^8 bacteria, and an unchallenged control group was also included, administered sterile saline. The identification of the animals in each challenge group was 1578N + 1638N, 1630N + 1570N, 1549N + 1531N, 1689N + 1728N, and 1640N + 1603N, respectively. For challenge, a 1 ml bacterial inoculum was administered subcutaneously, 2 cm caudal to the base of the left ear of each animal, on a line passing through the ear to the left eye. Subsequently, animals were housed in their respective groups for nine weeks, during which time blood was collected at regular intervals for serology. Animals were then sacrificed, a full necropsy examination was performed, and samples corresponding to inoculation site abscesses, lung, liver, spleen and brain tissues, as well as left- and right-hand-side (LHS and RHS) parotid, prescapular, sub-mandibular, retropharyngeal, mediastinal, prefemoral, inguinal and popliteal lymph nodes were collected for determination of the presence of bacteria.

For immunisations, vaccine antigens were formulated in physiological saline to final volumes of 1 ml, containing 50% (v/v) of a 13 mg/ml colloidal suspension of aluminium hydroxide (Alhydrogel; Sigma). Groups of six animals were immunised with either saline (group 1; control), 50% Alhydrogel (group 2; control), 50 μ g of rPLD (group 3), 5×10^9 formalin-killed *C. pseudotuberculosis* whole-cells (group 4), or a combination of 50 μ g of rPLD and 5×10^9 killed whole-cells (group 5). Group 2 was included to ensure that there was no effect of the adjuvant on the host resistance to infection. In addition, animals were immunised with the commercial inactivated-PLD vaccine, Glanvac-3™

(Commonwealth Serum Laboratories (CSL) Ltd., Victoria, Australia), currently unlicensed in the UK (Group 6). Vaccines were administered sub-cutaneously to the RHS lateral neck. A four week interval was allowed between the first and second immunisations. Subsequently, vaccinated animals were challenged with the determined optimal bacterial dose as described earlier in this section, following which they were housed in their respective groups for three months until postmortem examination.

(h) Post mortem bacteriological analyses

To determine infection with *C. pseudotuberculosis*, the post mortem tissue samples listed in section (g) were macerated in PBS using a stomacher (Wolf Laboratories Ltd, York), and aliquots were then plated onto BA plates, and incubated to allow colony development. Presumptive identification of recovered bacteria as *C. pseudotuberculosis* was made on the basis of colony morphology, catalase activity, Gram-stain and appearance by microscopical analysis. A random selection of samples was also analysed using the API Coryne test (bioMérieux).

(i) Serology

Animals' immunological responses to vaccination and/or infection were determined by measurement of serum anti-*C. pseudotuberculosis* and anti-PLD IgG by ELISA. Ninety-six well microtitre plates were coated with either rPLD (rPLD-ELISA) or whole *C. pseudotuberculosis* cells (WC-ELISA) and blocked with 1× TBS buffer containing 0.05% (v/v) Tween 20 (TBST) and 3% (w/v) Top-Block (Sigma). Subsequently, plates were incubated in the presence of experimental serum samples, diluted (1/200) in TBST containing 1% Top-Block. Plates were washed with TBST, and bound anti-*C. pseudotuberculosis* and anti-rPLD IgG was detected by use of a horseradish peroxidase-conjugated mouse anti sheep/goat IgG monoclonal antibody (Clone GT-34; Sigma) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma). Colorimetric reactions were stopped and intensified with 2 M H₂SO₄, and the absorbance of each sample at 450nm was subsequently determined.

(j) Computational and statistical analyses

Analysis of DNA sequences and design of oligonucleotide primers was performed using the Clone Manager Professional Suite (Scientific and Educational Software Ltd., Durham, NC, USA). Statistical analyses were performed using Genstat (8th edition; VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Because of the lack of independence between measurements made on the same animal, a repeated measures model was fitted to the rPLD IgG ELISA data. The correlation structure was modelled using a power model and the parameters of the statistical model were estimated using the restricted maximum likelihood directive in Genstat. Several analyses were carried out on the necropsy examination data to determine differences in the extent and pattern of infection in the various tissues. A one-way analysis of variance (ANOVA) was used to investigate the mean percentage of tissues infected with *C. pseudotuberculosis* in the different groups. An angular transformation of the percentages was used to stabilise the variances within the groups and mean differences between groups were assessed using a least significant difference (LSD) approach. Three different patterns of infection were defined: no tissues infected; infection at the inoculation site only; and infection at the inoculation site and at least one other tissue. Animals were classified as having one of these three pattern types and a Fisher exact test was performed to determine whether there was an association between groups and pattern types. The nature of the association was illustrated using the statistical methodology correspondence analysis.

Results

(a) Optimisation of infection model

In order to determine the optimal dose of *C. pseudotuberculosis* required to induce a similar disease manifestation in experimentally-infected animals to that observed in natural cases of CLA, a dose titration experiment was performed. Prior to infection, the absence of clinical symptoms and of circulating anti-*C. pseudotuberculosis* whole-cell or anti-PLD IgG was confirmed by clinical examination and ELISA analysis of blood

samples taken from experimental candidates using the rPLD- and WC-ELISAs (Figure 7.1). Thus, the absence of natural infection within the flock was established. Bacteriological analysis of tissue samples taken *post mortem* was performed, and the sites from which *C. pseudotuberculosis* was recovered from each animal are presented in Table 7.2. In light of the observation that no bacteria were recovered from either brain, spleen or liver tissue samples or inguinal, popliteal and prefemoral lymph nodes from any of the experimental animals in the current study or in infection experiments conducted previously (unpublished data), subsequent experiments were refined by eliminating the sampling of these sites. From the bacteriological data, it was apparent that as few as 10^2 cells were capable of causing infection. A challenge dose of 10^4 cells was chosen for subsequent experiments, due to the observation that, with this dose, infection occurred in multiple lymph nodes and the lungs (*i.e.* was not restricted to one specific site).

Serological analysis of the blood samples taken from the experimental animals during the course of infection was performed. The levels of anti-*C. pseudotuberculosis* whole-cell and anti-PLD IgG of each animal were determined, and are presented in Figure 7.1 as the absorbance values at 450nm (OD_{450nm}), obtained using either ELISA. As expected, all infected animals were observed to have mounted an immunological response to the challenge bacterium, as determined using the WC-ELISA; however there was no evidence that the magnitude of the response was directly associated with the number of challenge bacteria administered. Results were similar for anti-PLD IgG determination using the rPLD-ELISA. However, it is interesting to note that, despite mounting an immune response against *C. pseudotuberculosis* whole cells, one animal (1531N) failed to mount any observable response to PLD during the time-course of the experiment (see Figure 7.1).

(b) Vaccination and challenge of experimental animals

Prior to vaccination, experimental candidates were screened by clinical examination and by both ELISAs; all animals were free from observable signs of infection and lacked

circulating anti-*C. pseudotuberculosis* whole-cell and anti-PLD IgG. Unfortunately, one animal in group 3 died unexpectedly in the period between the first and second immunisations. No inoculation site reaction was observed, and it was determined that cause of death was not associated with any effects of vaccination.

Prior to experimental challenge, serology was performed to monitor the response to vaccination in each animal, using the rPLD-ELISA and WC-ELISA (Figure 7.2, top and bottom panels, respectively). To compare immune responses between treatment groups, data are presented as average OD_{450nm} values (as determined by either ELISA) per group, and standard errors at each time point are included. As expected, using the rPLD-ELISA, the highest levels of anti-PLD IgG were observed for the animals immunised with rPLD (groups 3 and 5) and Glanvac™ (group 6). For groups 3 and 5, anti-PLD IgG levels reached a maximum at the point of the booster vaccination. Furthermore, given the similar responses in animals in these groups, the whole-cell portion of the vaccine administered to group 5 was not considered to contribute to the immune response measured using the rPLD-ELISA. Levels of anti-PLD IgG in Glanvac™ vaccinates were consistently less than those observed for groups 3 and 5, and, rather than peaking, continued to rise following the secondary vaccination.

A repeated measures model was fitted to the rPLD IgG ELISA data. There was strong evidence ($P < 0.001$) of a different pattern in the responses over time, particularly where the levels of anti-PLD IgG in groups 3 and 5 were significantly higher than those of group 6 in the weeks immediately post-immunisation and pre-challenge (see Figure 7.2). Levels of anti-PLD IgG in animals immunised with formalin-killed whole-cells (group 4) were similar to those of non-immunised animals (groups 1 and 2), confirming the observation that the immune response measured in group 5 animals was likely to be as a result of the rPLD portion of the vaccine.

In contrast to results using the rPLD-ELISA, the WC-ELISA was able to determine the presence of high levels of anti-*C. pseudotuberculosis* IgG in killed whole-cell-

vaccinated animals (groups 4 and 5), while not detecting an immune response in those animals vaccinated with rPLD or Glanvac™ (groups 3 and 6). This was probably due to the fact that PLD is a secretory antigen, which would have been sparingly present in the killed whole-cell vaccine, and also in the cell preparations used in the WC-ELISA. Therefore, given the similarities in IgG levels between groups 4 and 5, the response in group 5 can only have been against the whole-cell portion of the vaccine. As with groups 3 and 5 (using the rPLD-ELISA), antibody levels in groups 4 and 5 reached their maximum level prior to administering the secondary vaccination. Overall, using the combined results of the two ELISAs, it was possible to determine that all animals, except for those of the control groups, had mounted observable immunological responses to vaccination.

The levels of serum anti-PLD and anti-*C. pseudotuberculosis* IgG were determined, as for the pre-challenge serum samples, following bacterial challenge at week six (Figure 7.2). After challenge, average anti-PLD IgG levels in the control groups (1 and 2) increased to their maximum level by week ten (four weeks post-challenge). Anti-PLD IgG levels in killed whole-cell vaccinates (group 4) remained similar to those of the control groups, rising steadily to a maximum level by week 10. Anti-PLD IgG levels in groups 3 and 5 remained essentially constant until week 10, by which point anti-PLD IgG in group 6 animals had reached an equivalent level. From that point on, anti-PLD levels in groups 3, 5 and 6 declined gradually to coincide with the anti-PLD levels observed in animals of groups 1, 2 and 4. Levels of anti-*C. pseudotuberculosis* IgG in groups 4 and 5 remained essentially constant from the point of challenge. Anti-*C. pseudotuberculosis* IgG levels in groups 1, 2, 3 and 6 were similar to each-other, rising slightly after challenge, but never reaching an equivalent level to that observed for killed whole-cell vaccinates.

(c) *Postmortem examination*

Postmortem examination and bacteriological sampling of tissues from vaccinated experimental animals yielded significant findings. The numbers of infected sites varied

between groups, and to a lesser extent between individual animals. However, *C. pseudotuberculosis* was not recovered from the RHS parotid or the LHS and RHS submandibular lymph nodes of any animal in the study. Data pertaining to the loci within animals of each treatment group from which bacteria were isolated is presented in Table 7.3. Significantly, no evidence of infection was found within any of the animals in group 5, including the apparent resolution of inoculation site abscesses. The observed number of lung lesions varied to a large extent, both between groups and individual animals. Therefore, rather than directly comparing the actual numbers of lung lesions as a means of determining disease severity, a more definitive method of comparing the presence or absence of lesions was used to determine differences, as follows below.

An ANOVA was performed to determine whether the mean percentage of tissues infected with *C. pseudotuberculosis* varied significantly between the different treatment groups. Only the eight tissues observed to show infection in any of the animals were used in the analysis (RHS parotid excluded). There was strong evidence ($P < 0.001$) that the mean percentage of tissues infected was different between the different treatment groups. The mean percentages and standard errors on the original scale are shown in Table 7.4. Groups 1 and 2 had similar means, and there was no evidence of an effect of Alhydrogel on the outcome of infection. Furthermore, groups 1 and 2 were both significantly different from groups 3, 4, 5 and 6 (using an LSD at the 5% level), confirming that all of the vaccine antigens assessed in the study offered a significant level of protection against infection.

In the experimental subjects, the most commonly encountered site of infection was the LHS prescapular and retropharyngeal lymph nodes, which is consistent with the drainage of challenge bacteria from the site of administration to a local draining lymph node. To determine whether spread of *C. pseudotuberculosis* from the inoculation site to other internal sites was more or less likely within the different treatment groups, each animal was assigned to one of three different pattern groups, which were determined on the basis of there being no tissues infected (P1), infection in the inoculation site but no

other tissues (P2), infection in the inoculation site and at least one other tissue (P3). A Fisher exact test indicated that there was very strong evidence ($P < 0.001$) that some treatment groups displayed different patterns of infection to other groups.

The association between treatment group and lesion pattern type was investigated further using correspondence analysis (see Figure 7.3). The scores of the first and second components were plotted against each other for treatment groups and lesion pattern types separately. It was expected that if a pattern type (P1, P2 or P3) was associated with a particular treatment group (G1-6) then the pattern and treatment groups would be located close to each other on the correspondence plot. It was observed that P1 and G5 were clearly associated with each other and separated from the other groups on the first component. Furthermore, on the second component, P2 and P3 were distinct, with G3 and G4 associated with P2 and G1 and G2 associated with P3. Interestingly, G6 did not cluster well with any pattern group, but was most closely associated with P3.

Table 7.1 Oligonucleotide primers produced for use in these studies.

Primer Name	Sequence ^a	Description
<i>pld#01b</i>	5'-CGCGCCATATGGCGCCTGTTGTGCATAACCCAGC-3'	Binds at bp 73-95 of <i>pld</i>
<i>pld#02</i>	5'-GCGCGGGATCCTCACCACGGGTTATCCGC-3'	Binds at bp 924-907 of <i>pld</i>
pET(fwd)	5'-GGATAACAATTCCTCC-3'	Binds at bp 5,261-5,277 of pET-15b
pET(rev)	5'-CTAGTTATTGCTCAGCG-3'	Binds at bp 5,452-5,436 of pET-15b

^aRestriction endonuclease cleavage sites underlined; *pld#01b*=*NdeI* and *pld#02*=*Bam*HI.

Table 7.2 Recovery of *Corynebacterium pseudotuberculosis* from tissues of challenged animals.

Dose (cfu/ml)	Animal #	Tissue sample ^{a,b}						
		Lung	LPs.	LPa.	LR	Med	IS	
Control	1640	-	-	-	-	-	-	-
Control	1603	-	-	-	-	-	-	-
10 ²	1579	+	-	-	-	-	-	+
10 ²	1638	+	-	-	-	-	-	+
10 ³	1630	+	+	+	+	-	-	+
10 ³	1570	+	-	-	+	-	-	+
10 ⁴	1549	+	+	-	-	+	+	+
10 ⁴	1531	-	+	+	-	+	+	+
10 ⁸	1689	+	+	-	+	+	+	+
10 ⁸	1728	+	+	-	+	+	+	+

^aLPs.=left hand side (LHS) pre-scapular lymph node; LPa.=LHS parotid lymph node; LR=LHS retropharyngeal lymph node; Med=mediastinal lymph node; IS=inoculation site. ^bPresence/absence of bacteria denoted by +/- respectively.

Table 7.3 Recovery of *Corynebacterium pseudotuberculosis* from tissues of vaccinated, challenged animals.

Group No.	Group size	Treatment	Tissue sample ^a							
			IS	LPa	LPs	RP _s	LR	RR	Lung	Med
1	6	Saline	6/6	1/6	3/6	1/6	3/6	2/6	1/6	1/6
2	6	Alhydrogel	6/6	2/6	5/6	0/6	4/6	0/6	3/6	1/6
3	5	rPLD	5/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5
4	6	Whole-cell	5/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
5	6	rPLD + whole-cell	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
6	6	Glanvac TM	5/6	1/6	1/6	0/6	3/6	0/6	0/6	0/6

^aAbbreviations correspond to: tissues; **IS**, inoculation site, and lymph nodes; **LPa**., LHS parotid; **LPs**., LHS prescapular; **RP_s**., RHS prescapular; **LR**, left retropharyngeal; **RR**, right retropharyngeal; **Med**, mediastinal

Table 7.4 Percentage of infected tissues by treatment group.

	Treatment group					
	1	2	3	4	5	6
Group size (n)	6	6	5	6	6	6
Mean^a	37.5	43.7	15.0	10.4	0	20.8
Standard error	6.5	8.4	2.5	2.1	0	7.0

^aMean percentage of tissues containing CLA lesions.

Figure 7.1 Measurement of humoral immune response in sheep challenged with *C. pseudotuberculosis*. Levels of IgG (presented as OD_{450nm} values) against *C. pseudotuberculosis* whole-cells (top) and rPLD (bottom) were determined. Challenge was performed at week 0, and groups correspond to: Control, 1640N (—) and 1603N (—×—); 10², 1638N (---▲---) and 1579N (---△---); 10³, 1570N (—●—) and 1630N (—○—); 10⁴, 1531N (—■—) and 1549N (—□—); 10⁸, 1728N (---◆---) and 1689N (---◇---).

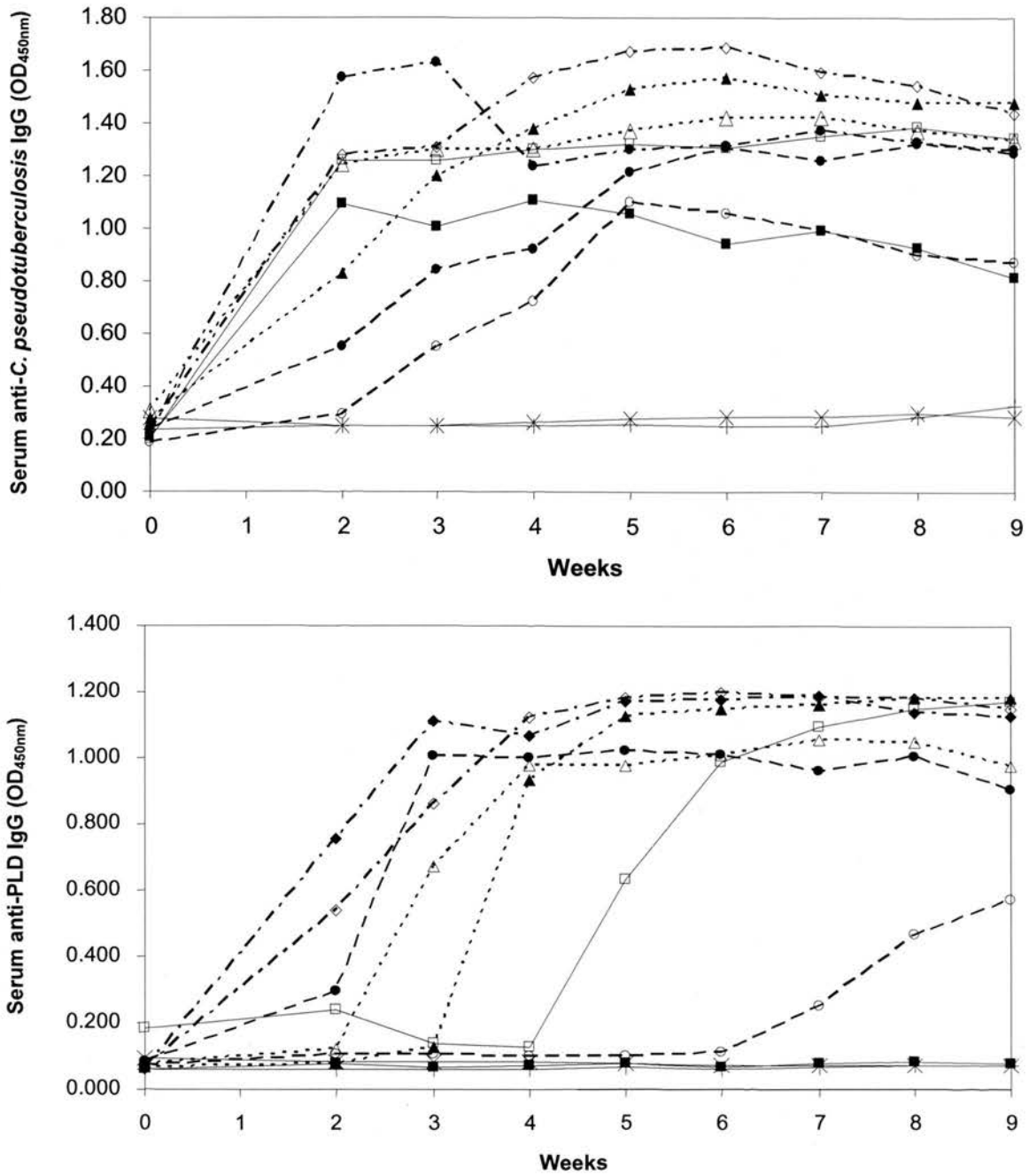


Figure 7.2 Measurement of anti-PLD and anti-*C. pseudotuberculosis* IgG.

Serum anti-PLD (top) and anti-*C. pseudotuberculosis* whole cell (bottom) IgG levels were calculated for each treatment group, expressed as the mean and standard error of OD_{450nm} per group. Samples were obtained following primary and secondary vaccinations (weeks 0 and 4 respectively) and for 3 months following bacterial challenge (at week 6). Treatment groups are 1. (—◆—) saline control, 2. (- -■ - -) Alhydrogel control, 3. (- -△ - -) rPLD, 4. (- * -) killed-whole cells, 5. (—▲—) rPLD + killed-whole cells, and 6. (—○—) Glanvac-3.

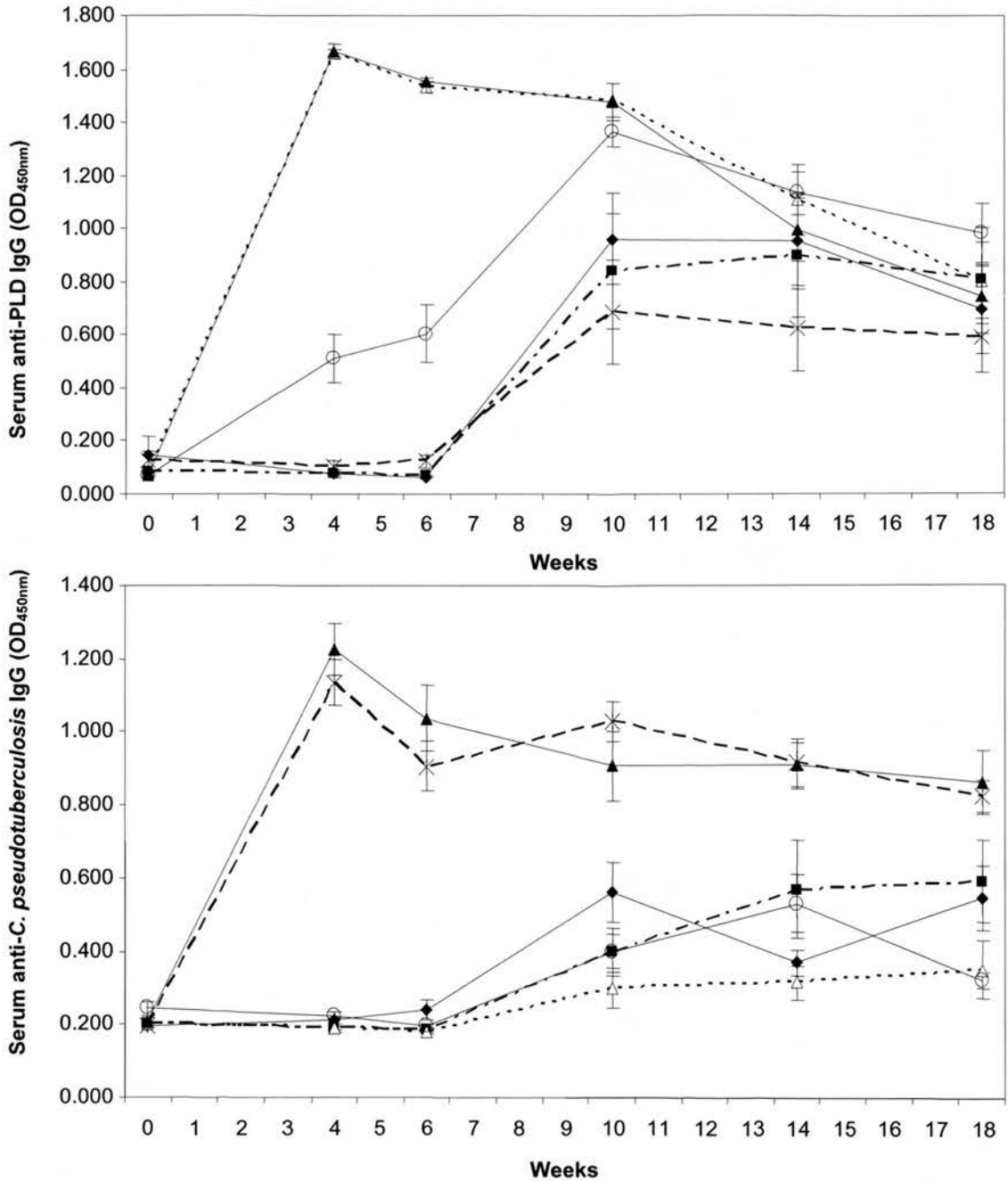
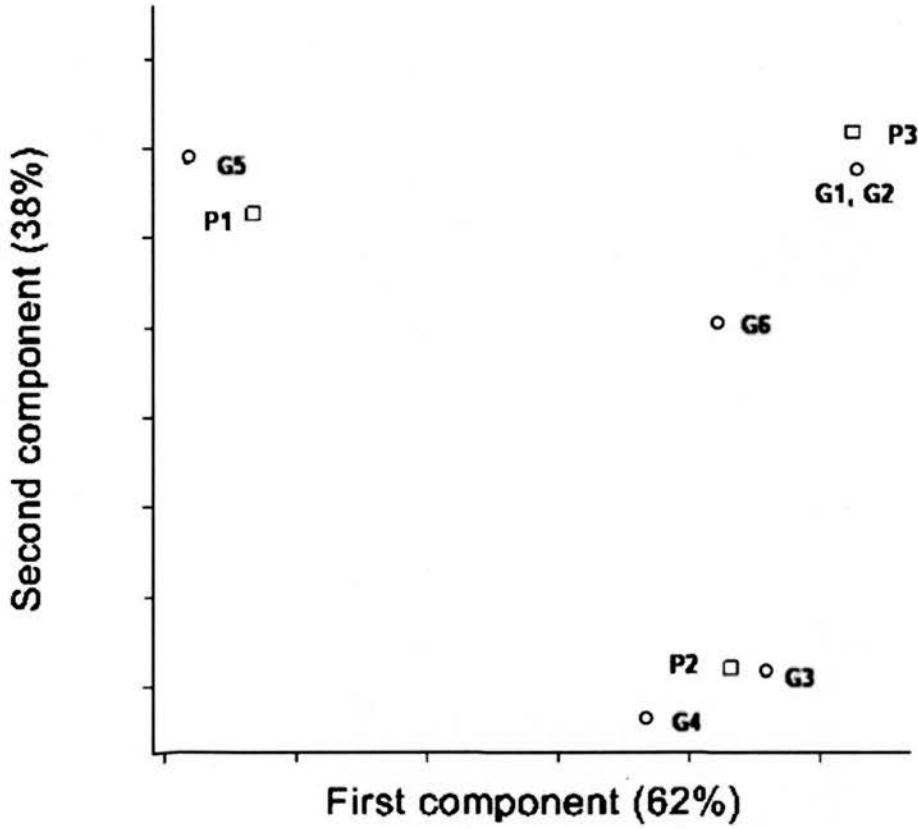


Figure 7.3 Correspondence analysis of treatment groups and pattern of infection. Treatment groups were saline (G1), Alhydrogel (G2), rPLD (G3), killed whole-cells (G4), rPLD + killed whole-cells (G5) and Glanvac-3 (G6). Patterns of infection were defined as no tissues infected (P1), infection in the inoculation site but no other tissues (P2) and infection in the inoculation site and at least 1 other tissue (P3). All treatment groups associate with a particular pattern of infection, with the exception of G6.



Discussion

This is the first UK study to consider the vaccination of sheep against CLA and confirms statistically significant levels of protection against challenge with a UK-derived *C. pseudotuberculosis* strain. In the homologous challenge model, vaccination with either the highly-purified rPLD antigen, or formalin-killed *C. pseudotuberculosis* cells, prevented spread of bacteria beyond the site of inoculation in the majority of immunised animals. More importantly, vaccination with a combination of rPLD and killed whole-cells resulted in complete protection against challenge, inasmuch as no evidence of infection was identified in any of the tissue samples from challenged animals. Vaccination with the commercial vaccine, Glanvac™, also offered significant protection against heterologous challenge, although it was less effective in preventing the dissemination of infection beyond the site of inoculation.

The experimental infection model that was used produced a pattern of lesions in sheep that was consistent with clinical CLA as recognised in the UK (Rizvi and others 1997; Malone and others 2002). The initial dose of bacteria used was chosen to be high enough to ensure that infection was consistently achieved, but low enough to avoid any possibility that the host immune response might be overwhelmed, thereby masking any protective effects of vaccination. This is important since theories of CLA pathogenesis suggest that natural infection may be initiated by small numbers of aerosol-borne bacteria (Pepin and others 1994a).

Earlier studies have considered different forms of PLD-based vaccine capable of inducing protective immunity by stimulating production of anti-PLD antibodies (Batey 1986c; Brown and others 1986; Eggleton and others 1991a; Eggleton and others 1991b; Sutherland and others 1992; Hodgson and others 1999), with no apparent effect on cell-mediated immunity (Hodgson and others 1999). In this trial a highly-purified recombinant derivative of PLD was used. Of the five rPLD immunised sheep (group 3), dissemination of the challenge bacteria beyond the site of inoculation was prevented in all but one individual that manifested evidence of lung infection.

In this study the animals vaccinated with rPLD mounted a rapid antibody response, which peaked around the time of the second immunisation (Figure 7.2). In contrast, antibody response to the PLD contained within Glanvac™ corresponds to earlier observations (Hodgson and others 1994; Chaplin and others 1999) where anti-PLD IgG rose relatively modestly with successive vaccinations, but increased further following challenge. The reason for this statistically significant difference between vaccines may relate to the relative amounts of rPLD and wild-type PLD in the experimental and Glanvac™ vaccines, respectively.

To date, evidence for the protective effects of autogenous CLA vaccination in the UK has been purely anecdotal. However, the study demonstrated significant protection against homologous challenge using a *C. pseudotuberculosis* bacterin vaccine. Vaccination with killed whole-cells was sufficient to prevent the spread of *C. pseudotuberculosis* beyond the site of inoculation, and in one animal even the inoculation locus was found to be sterile.

The genome of *C. pseudotuberculosis* of sheep and goat origin from the UK and other countries would appear to be extremely stable (Connor and others 2000; Connor and others 2007). However, PFGE analysis of multiple *C. pseudotuberculosis* isolates from a CLA-affected flock has revealed the presence of different, albeit related, clones in different animals (M. C. Fontaine, personal communication). This has important implications for the success of autogenous vaccination strategies, should they be found not to confer protection against heterologous *C. pseudotuberculosis* strains.

These results support previous findings that neither rPLD nor whole cell vaccine was sufficient on its own to resist artificial infection (Cameron and others 1972; Cameron and Fuls 1973). In contrast, immunisation of sheep with a combination of rPLD and formalin-killed whole-cells produced an apparently complete protection against experimental challenge. Even the infected sites of inoculation, which frequently contained live *C. pseudotuberculosis* cells in other experimental groups, completely

resolved and were found to be sterile on culture. These observations are consistent with those of a previous study (Burrell 1983).

C. pseudotuberculosis has been shown to survive killing by non-activated macrophages of several species (Hard 1972; Tachedjian and others 1995) and the importance of activated macrophages in the suppression of *C. pseudotuberculosis* growth and lesion development has been shown in a murine model (Hard 1970; Simmons and others 1997). In addition, murine macrophage killing of *C. pseudotuberculosis* following opsonisation with hyperimmune ovine serum has been demonstrated (Hard 1972). Taken together, these observations may help to explain the effectiveness of the combined rPLD/whole cell vaccination. Neutralisation of PLD (and other *C. pseudotuberculosis* proteins) by serum antibodies and opsonisation of bacteria for killing by non-activated macrophages, would be combined with priming of the cell-mediated immune response, thus allowing activated macrophages to more effectively kill introduced bacteria. It should be noted however that no attempt was made to measure a possible cell-mediated immune response during any stage of the experiment.

While the protection offered by Glanvac™ was lower than with the other experimental vaccines tested, it is of relevance that this was the only vaccine tested for its capacity to protect against heterologous challenge. For this reason, direct comparison between the protective capacity of Glanvac™ and the rPLD vaccine is not possible. It is noteworthy that although the number of infected loci was reduced in Glanvac™ immunised animals, there was only one animal which appeared completely free from infection. In contrast to observations of the rPLD and killed whole-cell vaccines, immunisation with Glanvac™ was not effective in preventing the dissemination of *C. pseudotuberculosis* beyond the primary site of infection.

Interestingly, no lung lesions were identified in Glanvac™-vaccinated animals. An aerosol route of *C. pseudotuberculosis* transmission has previously been postulated (Stoops and others 1984), and it is therefore possible that, despite not completely preventing infection, the removal of a potential route of disease transmission could

have a significant impact on the spread of infection to other animals. This hypothesis is consistent with the findings of an Australian study, in which Glanvac™ vaccinated sheep with CLA were observed to have 96% fewer lung abscesses compared with unvaccinated infected sheep (Paton and others 1995). In that study, 77% of disease transmission was found to occur around the fourth or fifth shearing, and in the absence of obvious superficial discharging abscesses, implying spread from discharging lung abscesses.

One question that remains unanswered is whether the difference in protection between Glanvac™ and the experimental vaccines was simply due to issues with the heterologous versus homologous challenge models, or due to the fact that Glanvac™ was simply outperformed by the experimental vaccines. This is relevant, since a single vaccine, if chosen wisely, might have the capacity to protect against infection by a variety of different isolates, irrespective of their geographical origin. Clearly, protection against heterologous challenge by the experimental vaccines needs to be determined, and future experiments would be required to address this.

Chapter 8: CASEOUS LYMPHADENITIS IN THE UK – JUSTIFICATION FOR AND MEANS OF CONTROL

While the commonly accepted view is that *C. pseudotuberculosis* infection arrived in Britain with a single consignment of goats in September 1987 (Meldrum 1990), it is impossible to substantiate this suggestion. Indeed, tracings carried out after the first isolation of the organism from a homebred sheep, failed to indicate any obvious links with recently imported animals (Robins 1991), suggesting that the infection may have been present in the UK rather longer than appreciated. What does appear indisputable, is that *C. pseudotuberculosis* was absent from the UK until the late 1980's. Consequently, the emergence of CLA within the domestic sheep industry over the last 20 years represents a national epidemic, albeit one occurring at a relatively slow pace.

The initial spread of CLA was undoubtedly assisted by a lack of awareness of this novel condition by farmers and veterinary surgeons, combined with the sub-clinical nature of many CLA cases. This was exacerbated by established practices of animal trade and movement within the industry, which enabled infection to spread widely around the country.

As discussed in Chapter 3, this epidemic was at first restricted to the terminal sire sector of the industry. That study showed clearly how far infection had extended in the ten years after the first diagnosed case of CLA in a UK sheep, with some 18% of sampled flocks showing serological evidence of infection. Given the stratified nature of the British sheep industry, the presence of any disease in flocks at the top of the breeding pyramid is cause for concern, and recent events have tended to confirm fears that infection would spread from these elite flocks to the wider commercial sector.

CLA has now been identified as a problem in some hill and upland breeds, with several outbreaks being reported in North Country Cheviot flocks in the north of Scotland (Anon 2004). As indicated in Chapter 6, CLA is a disease quite capable of

spreading effectively within flocks managed in much more extensive systems. This is consistent with findings from Australia where CLA is highly prevalent in extensively managed flocks that are maintained at stocking densities broadly equivalent to that of many flocks in this country (Paton 1997).

Based on experience gained in this country over the last 20 years, and on the epidemiology of the infection in other parts of the world, the assumption for the UK must be that if left unchecked, CLA will become a highly prevalent disease within the sheep industry as a whole. Given that CLA is already effectively enzootic within Britain, the question becomes one of whether disease control is worthwhile, and if so, how this might best be achieved.

Why is control necessary?

It has been argued that whilst CLA is contagious and aesthetically unpleasant, its relatively mild systemic effects do not justify significant expenditure of time and resources in its control. It has also been suggested that since *C. pseudotuberculosis* is prevalent world-wide, the presence of infection within the UK national flock will have little impact on this country's ability to trade, or place this country at an economic disadvantage when compared to competitor nations. This was the principal justification used by MAFF when it deregulated the emerging disease of CLA in the early 1990's (Robins 1991). Another argument used at the time, was that while *C. pseudotuberculosis* does possess a potential for zoonotic infection, this occurs infrequently, and does not justify significant expenditure to mitigate the risks. Indeed, in other countries the condition in man is seen mostly as an occupational hazard, and as such is best avoided by way of workplace health and safety measures on farms or in abattoirs (Peel and others 1997).

However, reference to the sheep industries in other parts of the world would tend to counter an argument to do nothing. In the majority of countries CLA is regarded as a problem worthy of control, usually by means of routine vaccination and the application of certain management practices. In Australia particularly, it has been recognised that although a minor condition for the majority of animals, the

cumulative financial effects of CLA on the national sheep flock, through decreased wool production and carcase condemnation, was significant (Paton 1997); and that this justified a disease control programme on economic grounds. Similar observations have been made in North America (Williamson 2001) and South Africa (Paton and others 2005).

Whilst CLA has been recorded in many different breeds and in all regions of the UK, it remains an uncommon diagnosis. Because of this, the disease carries a stigma amongst producers of pedigree breeding animals. Many such producers feel that the disease would place their enterprise at a competitive disadvantage, and are therefore anxious to avoid buying the problem into their flocks (Davies and Baird 2007). Recent concerns have also been voiced in the Netherlands following the importation of several consignments of UK Suffolk rams that were subsequently diagnosed with the condition (D. P. Dercksen, personal communication). The owners of UK flocks where CLA has already been diagnosed are similarly anxious that their sale animals are not sub-clinically infected. It was these considerations that motivated the owners of the flocks in Chapters 5 and 6 to regard CLA control as a priority.

Another driver for CLA control to emerge recently is the clinical similarity of the condition to tuberculosis (TB) due to *Mycobacterium bovis* in small ruminants. Outbreaks of TB have been diagnosed in goats (Crawshaw and others 2008; Daniel and others 2009) and sheep (Houlihan and others 2008) in recent years, and have caused some alarm within the farming community. During the investigation of these outbreaks CLA was considered to be a differential diagnosis, based on the clinical signs of lymphadenopathy and ill thrift (Van Der Burgt 2010). In one case a concurrent TB and CLA infection was diagnosed in a herd of goats (Sharpe and others 2010). This led the authors of that report to warn practitioners to be aware of these similarities, and to urge them to suspect TB where serological or bacteriological evidence of CLA was lacking in a particular case.

The past two decades have shown us that where efforts at controlling CLA are absent, or at best limited to the culling of clinically affected animals, the spread of

infection is relentless. If control of CLA is accepted as being desirable, the optimal means by which this can be achieved then becomes important. The preceding chapters have described ways in which this objective might be reached through the use of serology and vaccination.

Serological testing

Test and cull programmes

Chapters 5 and 6 of this thesis describe the potential benefits of an ELISA-based test and cull programme for the control of CLA. Complete disease eradication is likely to be an achievable objective, albeit one that is immensely challenging, requiring significant organisational effort and the likelihood of substantial financial cost.

In recent years, work in the UK carried out by the author and colleagues has led to the development of a commercially available CLA ELISA kit (and a related Western blot assay based on the test recombinant PLD antigen) for use in sheep and other species (Anon 2007a). This assay was used in the trial reported in Chapter 6 and has similar levels of sensitivity and specificity to the ELISA used in the trial reported in Chapter 5. More than 10,000 ELISA tests were run for the purpose of CLA control and potential eradication in flocks within the UK during 2009. As anticipated, the expense associated with testing a large number of animals, means that the majority of this work is currently done within small flocks of high value pedigree sheep.

Pre-sale screening of breeding animals

A second way in which the CLA ELISA has been used in recent years is as a tool in the pre-sale screening of breeding animals. The CLA Monitoring Scheme was established by the author under the auspices of the Premium Sheep and Goat Health Schemes (Anon 2005). The basis of this scheme is the certification of animals that have undergone specified serological and clinical examinations during a three month quarantine period. Since the establishment of the programme in 2005, approximately 5,200 sheep have been tested within the CLA Monitoring Scheme. This represents around 300 groups, of mostly breeding rams, which were marketed as CLA-monitored at ram sales in Kelso (Roxburghshire), Builth Wells (Powys), Lairg

(Sutherland) and elsewhere. While the intention of this scheme was to introduce a level of quality assurance for buyers, with a potential for added value for sellers, it has not been widely supported by the terminal sire breed societies.

A local initiative that has gained considerably more support has been the screening for serological evidence of CLA of all sheep entering the Shetland Islands, which has been in operation since 2008. This scheme was established after two major flock outbreaks on the Islands (Anon 2007b, 2008) led to local concern on the part of local farmers, who import up to 500 rams each year. These animals are from the terminal sire breeds (mainly Suffolk) and are used for crossing with the indigenous Shetland sheep breed. The Shetland CLA scheme is enhanced by general acceptance from farmers, the single point of entry via the port of Lerwick and substantial financial backing from the local Council.

Vaccination

Chapter 7 describes how the use of vaccination can substantially prevent the development of clinical CLA in animals infected with UK isolates of *C. pseudotuberculosis*. Vaccination is the primary means of disease control in most major sheep producing countries, where immunisation reduces the spread of infection, and leads to a gradual decline in disease prevalence. However, no proprietary CLA vaccine currently available offers complete protection against infection by *C. pseudotuberculosis*; hence, animals will continue to develop clinical disease, albeit in reduced numbers. This is evidenced by a report from Australia which indicated that, despite the widespread use of a commercial CLA vaccine in that country since 1983, disease prevalence remained at approximately 20% in 2002 (Paton and others 2003). One explanation for this is provided by evidence that as few as 9% of producers may be employing CLA vaccination effectively (Paton 2010), a trend that might very well be repeated in the UK.

A hurdle that may now stand in the way of CLA eradication by means of ELISA testing is that of vaccination. Although as stated, no fully licensed vaccine is available in the UK, the use of vaccination against CLA is possible with the

authorisation of the VMD. With the agreement of VMD an autogenous *C. pseudotuberculosis* vaccine may be prepared in this country. Alternatively, on receipt of a Special Treatment Certificate from the VMD, a veterinary practitioner may on behalf of a client, import a proprietary CLA vaccine into the UK. However, anecdotal evidence suggests that unauthorised importation and use of proprietary vaccines may also be occurring. This is significant, since as described in Chapter 7, CLA vaccines containing PLD antigen have been shown to produce seroconversion detectable as positive titres in the ELISA test. For this reason the use of current vaccines is incompatible with a test and cull programme as described here.

Modelling disease control

Mathematical modelling techniques have now been applied to CLA epidemiology and control in the UK. In the first such model, data from four infected flocks was used to describe epidemic CLA and to estimate transmission coefficients for different routes of disease transmission (O'Reilly and others 2008). This model was then used to evaluate strategies for control of the disease and the potential for complete elimination of *C. pseudotuberculosis* from affected sheep flocks; the latter being defined as a 99% confidence that no sheep remained infected (O'Reilly and others 2010). The overarching conclusion of this work was that CLA control programmes should begin as soon as *C. pseudotuberculosis* infection is first confirmed. The model predicts that elimination of the infection is most likely using a serological test with sensitivity and specificity of at least 90%. This was deemed to be achievable after five tests, although with a significant impact on flock productivity likely due to the removal of seropositive animals. The model also predicted that a combination of vaccination and clinical examination would also reduce infection rapidly, but with less likelihood of total elimination of the pathogen.

Scientific contributions made by this work

It is the author's hope and belief that the work described in this thesis has advanced the understanding of CLA in the UK and provided guidance on how effective control may be established. In the authors view the most salient contributions have been:

1. The identification of high CLA prevalence within the terminal sire sector, at a time when the industry awareness of this disease was relatively low.
2. Confirmation that conventional dairy pasteurization protects consumers from potential *C. pseudotuberculosis* contamination in milk
3. The demonstration that a CLA control programme incorporating ELISA testing can, under certain circumstances, facilitate disease eradication.
4. The eventual commercialisation of a CLA ELISA assay and its use today in disease eradication and sheep health schemes.
5. A demonstration that vaccination can protect sheep in a UK infection model and that a combined recombinant PLD and bacterin vaccine is potentially the most effective product.

Remaining gaps in our knowledge

Work conducted by the research team of which the author was a member, indicated that the genome of *C. pseudotuberculosis* strains responsible for CLA in this country is largely identical to that of isolates from other parts of the world (Connor and others 2007). Consequently, an explanation other than bacterial strain differences must exist to account for the contrasting clinical presentation of CLA in the UK compared to other countries (as described in Chapter 2). Although the principles of CLA disease pathogenesis are now well established in the literature, there are as yet no satisfactory explanations of why lesion distribution varies in different parts of the world. Although certain theories have been suggested, questions over mode of transmission and common routes of infection in the UK remain to be answered before guidance on disease prevention can confidently be given.

As indicated by recent modelling work, complete eradication of CLA becomes more feasible as the specificity and sensitivity of the serological tests improve. Whilst currently available ELISA tests offer specificity in sheep that is significantly greater than the 90% specified by modelling, sensitivity falls below this level – a feature which it shares with most other recent tests. Further development in the area of serology – perhaps in uncovering other conserved antigens associated with *C. pseudotuberculosis* – is required if test sensitivity is to be improved.

Debate continues over the best methods of controlling CLA in the UK. Vaccination is the main strategy in most of the countries where CLA is endemic and many in the UK farming community advocate the licensing of a proprietary vaccine. However, at present there seems little likelihood that the relevant pharmaceutical companies will complete the work required to obtain a full licence in the UK. In any case, as discussed in Chapter 7, simply licensing the existing proprietary vaccines for use in this country may not provide the solution that many in the farming community might hope for. This leaves the use of those proprietary vaccines under emergency licence from the VMD and the use of autogenous vaccines as the only legal options currently available to UK producers.

Development of a novel CLA vaccine is proceeding in the UK and this provides some encouragement that vaccination could become a more serious option for CLA control in the future. Reports indicate that a novel product based upon a Scottish *C. pseudotuberculosis* isolate is to be manufactured as a marker vaccine (Anon 2009b). This would in theory enable farmers to differentiate between serological responses produced in natural CLA infections and those resulting from vaccination. If successful that would enable a combined serological and vaccination approach to disease control, which would be expected to offer greater flexibility and significant benefits over either approach on its own. Further work and the agreement of the regulatory authorities would be required to enable these developments to become useful in the field.

Outlook for the UK

It is the author's opinion, based on the work described in this thesis, that accurate serodiagnosis together with segregation or culling of infected animals, currently offers a method for the complete eradication of CLA. The demonstration that vaccination can protect sheep from the UK form of disease offers further hope that the condition may be preventable in the wider sheep industry. Any future improvements in serodiagnosis and vaccination will require continued effort to understand the mechanisms by which *C. pseudotuberculosis* interacts with its host,

causes disease, and often persists throughout life. The story of CLA over the last 20 years has provided the UK with an object lesson on how an animal infection can progress from being a minor epizootic to a nationally enzootic disease. Whether the future is one of continuing disease spread or of eradication and control, will depend largely on whether the lessons learnt in the laboratory can be effectively communicated to and applied by, the farming industry.

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REVIEW

Corynebacterium pseudotuberculosis and its Role in Ovine Caseous Lymphadenitis

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Summary

Caseous lymphadenitis (CLA) of sheep, caused by *Corynebacterium pseudotuberculosis*, has been a significant disease in the majority of sheep-rearing regions for over a century. Because of the chronic and often sub-clinical nature of the infection, it has proved difficult to control and prevalence is high in many parts of the world, which in turn leads to significant economic losses for farmers. This review describes the important characteristics of *C. pseudotuberculosis* and examines the pathogenesis and epidemiology of the infection in sheep. The review also discusses the immune response to infection and describes the methods that have been developed to control CLA, with particular emphasis on the use of vaccination and serological testing.

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Keywords: bacterial infection; caseous lymphadenitis; *Corynebacterium pseudotuberculosis*; review; sheep

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Introduction

The perceived importance of caseous lymphadenitis (CLA) as a disease of sheep varies greatly around the world. Thus, in the UK, where this disease appeared for the first time in the early 1990s, the steady and apparently relentless spread of infection in sheep and goats has prompted great concern amongst both farmers and veterinary surgeons. However, in the sheep industries of Australia and New Zealand infection is regarded as relatively minor, albeit widespread. The roots of these contrasting attitudes lie in differences in disease prevalence, available means of control, and meat inspection practices.

To the veterinary pathologist, the causative organism of CLA, *Corynebacterium pseudotuberculosis*, has a valid claim to being the “perfect parasite”. Once successfully established within the host, this pathogen will evade the immune system with apparent ease. As a result, chronic infections may last for most or all of an animal’s life, although they are rarely fatal. If left unchecked, the disease may infect the majority of sheep in a flock. Even away from its mammalian host, the organism is well equipped for long-term survival in the environment. As a result of these formidable characteristics, flock infections by *C. pseudotuberculosis* are generally “managed”, rather than eradicated.

History

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

Towards the end of the 19th century the bacterium was described by the German bacteriologists Lehmann and Neumann in the first edition of their bacteriological atlas (Lehmann and Neumann, 1896). In that publication, the Preisz–Nocard bacillus was renamed *Bacillus pseudotuberculosis*—a derivation of the Greek *pseudes tuberculosis* or “false tuberculosis” and a reference to the supposed clinical similarity of the lesions to caseous nodules of mycobacterial tuberculosis. In the First Edition of Bergey’s *Manual of Determinative Bacteriology*, published in 1923, the organism was placed in the *Corynebacterium* genus, which had originally been created as a category for the human pathogen *Corynebacterium diphtheriae*. This edition of the *Manual* referred to work showing that *B. pseudotuberculosis* resembled *C. diphtheriae* in morphology and cell wall composition, leading

to a further change of name to *Corynebacterium ovis*. Subsequently, however, the organism was isolated from purulent infections and ulcerative lymphangitis in other mammalian species, including goats, horses and human beings. In recognition of this, by the time the Sixth Edition of Bergey’s *Manual* was published in 1948, the species name had been changed back from *ovis* to the earlier designation of *pseudotuberculosis*. Since that point, the officially recognized designation of *Corynebacterium pseudotuberculosis* has remained consistent (Euzéby, 2005), notwithstanding the fact that several authors continued to refer to the organism as *C. ovis* until the 1980s.

Geographical Distribution

The global range of *C. pseudotuberculosis* reflects that of farmed small ruminants, the bacterium having been identified in Europe, Australasia, North and South America, Africa and the Middle East (Robins, 1991; Paton *et al.*, 2005). In many of these countries CLA has been an established and economically important infection of livestock, particularly sheep, for decades. As early as the 1930s it was acknowledged that the disease “very extensively affected” the flocks of most mutton-exporting countries (Cesari, 1930). It is surprising therefore, that the first diagnosed case in an animal originating from the UK was not confirmed until as recently as 1989 (Lloyd *et al.*, 1990; Meldrum, 1990).

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

CLA first became a significant political issue in the 1920s when mutton carcasses, imported into Great Britain from a number of countries, were frequently found to be affected by the disease. It was noted at the time that evidence of infection would often remain unobserved at import meat inspection “only to be revealed when the roast leg of mutton appears on the table and is cut through” (Rolleston and Wooldridge, 1926). Mutton from Argentina was particularly badly affected,

forcing the British Government of the time to take action in relation to consignments of carcasses from that country (Anon, 1929). The extent of the problem was demonstrated by a report that in a single season 9770 mutton carcasses (representing 27% of the total throughput) were rejected at one Patagonian meat plant because of CLA (Mills, 1928). Other mutton-exporting countries took heed of the concerns in Britain and it was soon acknowledged that "Argentina, Uruguay, Chili (sic), Australia and New Zealand are actively occupied...in a struggle against this tenacious chronic disease which, completely neglected up to now, is very extensively affecting the flocks of those countries" (Cesari, 1930). These concerns also stimulated veterinary research, principally in Australia, leading to important early discoveries on pathogenesis, bacterial survival and risk factors (Seddon *et al.*, 1929; Bull and Dickinson, 1935).

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

Global Prevalence of CLA

C. pseudotuberculosis is recognized as having a worldwide distribution and it is accepted that CLA is present in the majority of the sheep-rearing areas. However, farm- and abattoir-based research aimed at establishing disease prevalence rates has been limited to relatively few countries. The average prevalence of CLA amongst adult sheep in Western Australia was recorded as 58% in 1973, and as 53% in 1984 (Batey, 1986a). In an Australian abattoir survey, 54% of adult ewes and 3.4% of lambs showed evidence of infection at meat inspection (Batey, 1986b). In another abattoir survey, individual flocks in Tasmania and Western Australia demonstrated prevalence levels within the adult population as high as 61% (Middleton *et al.*, 1991). Subsequent surveys tended to identify steadily decreasing prevalence rates, this generally being attributed to the introduction of a CLA vaccine in 1983 and its increasing acceptance within the farming community. A field study of 412 sheep flocks, once again in Western Australia, recorded an average prevalence of 45% (Pepin *et al.*, 1994a). A combined abattoir and postal survey of farmers, conducted in 2002, suggested that the average

prevalence had fallen to 20% in Western Australia, 23% in Victoria and 29% in New South Wales (Paton *et al.*, 2003).

A survey conducted in 1986/1987 by meat inspectors in New Zealand identified lesions of CLA in 7.1% of the adult sheep slaughtered and 0.64% of lambs (Nuttall, 1988). CLA was also identified as the leading cause of sheep carcass condemnation in South African abattoirs (Collett *et al.*, 1994) where losses of between 0.24% and 0.3% of all sheep carcasses were attributed to CLA and substantial additional losses were incurred due to carcass trimming (Paton *et al.*, 2005). In the western USA, average disease prevalence amongst adult ewes was estimated to be as great as 42.5% (Stoops *et al.*, 1984). Similar studies have been conducted in the Canadian province of Quebec, where the prevalence of clinical CLA was found to range from 21% to 36% amongst culled adult sheep (Arsenault *et al.*, 2003). Abattoir statistics from Alberta indicated that up to 5% of mutton carcasses and 0.03% of lamb carcasses were condemned due to CLA, and that a further 8% of all carcasses were trimmed to remove CLA lesions (Stanford *et al.*, 1998). Unfortunately, similar published statistics do not exist in respect of Europe. In the UK, where small abattoir surveys of CLA prevalence have been carried out, the results have been of limited scope and significance (Mechie, 1998).

Bacterial Characteristics

Classification

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

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

although it may appear only after incubation for 48–72 h.

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

The so-called “chemotaxonomic” approach to the classification of bacteria, based on analysis of the chemical composition of the cell wall, was at one time used relatively commonly. Analysis of cell wall mycolic acids greatly aided clarification of the taxonomy of actinomycetes, especially those of the genera *Corynebacterium*, *Mycobacterium*, *Rhodococcus* and *Nocardia* (Minnikin *et al.*, 1975; Goodfellow *et al.*, 1976; Keddie and Cure, 1977; Minnikin and Goodfellow, 1980; Collins *et al.*, 1982). In these studies, thin-layer (pyrolysis) chromatographic analysis of mycolic acid revealed that fatty acid chain length varied according to the genus and to a lesser extent the species. It was shown that mycobacterial mycolic acids normally consist of chain lengths of between 60 and 90 C₁₄ atoms, and may possess a number of distinct functional groups (Minnikin *et al.*, 1978). In contrast, nocardiae and rhodococci were shown to possess shorter mycolic acids, consisting of between 36 and 66 C₁₄ atoms, and with fewer functional groups (Minnikin and Goodfellow, 1976). The mycolic acids of corynebacteria were found to be even smaller, being between 20 and 36 C₁₄ atoms in length, and usually saturated or containing a single double bond (Minnikin *et al.*, 1978). Collins *et al.* (1982) reported that, consistent with the mycolic acid classification of corynebacteria, strains of *C. pseudotuberculosis* possessed mycolic acids with carbon chain lengths of between C₂₆ and C₃₆, which contained predominantly saturated C₁₄ side-chains (Collins *et al.*, 1982).

There are few published data on the resistance of *C. pseudotuberculosis* to chemical disinfectants. However, most common disinfectants, including calcium hypochlorite, formalin and cresol solution, appear to be effective in killing the organism, but the presence of organic material necessitates increased exposure time (Ismail and Hamid, 1972). This protective effect is clearly significant for a pathogen commonly found within a thick matrix of purulent debris. The organism is capable of surviving in commercial sheep dip solutions for 24 h or more, a point of relevance to disease control (Nairn and Robertson, 1974).

Early reports showed that *C. pseudotuberculosis* isolates from different mammalian species shared identical biochemical characteristics, with the exception of nitrate reduction. Thus, the majority of isolates from horses and cattle reduced nitrate to nitrite, while those from sheep and goats did not (Knight, 1969; Sutherland *et al.*, 1996). This led to the proposal of two distinct biotypes or subspecies (Biberstein *et al.*, 1971). Based on this property of nitrate reduction, Songer *et al.* (1988) proposed the designations *C. pseudotuberculosis* biovar *ovis* (biotype 1) and *C. pseudotuberculosis* biovar *equi* (biotype 2). However, the isolation in recent years of nitrate-negative strains of *C. pseudotuberculosis* from cattle (Yeruham *et al.*, 1997) and from horses (Connor *et al.*, 2000) suggests that such categorization may be unsatisfactory.

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

Virulence Factors

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

Phospholipase D. The designation “phospholipase” is used to describe a varied group of enzymes able to hydrolyse one or more ester linkages in glycerophospholipids; the letters A-D are used to distinguish between phospholipases and to denote the specific phospholipid ester bond that is cleaved (Ansell and Hawthorne, 1964). In eukaryotic cells, phospholipase enzymes play a role in signal transduction and normal membrane maintenance. Some mammalian cells also produce phospholipases as part of the cellular inflammatory response (Schmiel and Miller, 1999). Eukaryotic cell membranes are composed of proteins and lipids, which constitute a significant target of attack during microbial invasion of host tissues. As part of their invasive arsenal, many microbes have developed their own phospholipase enzymes, which may be used to hydro-

lyse phosphate bonds within membrane phospholipids (Ghannoum, 2000). The result of this is the damage or destruction of host cell membranes, which in turn may lead to their dysfunction or disruption, or both (Salyers and Witt, 1994). Various bacterial genera are known to secrete phospholipase enzymes, and in some cases these have been shown to play a role in virulence (Schmiel and Miller, 1999). Phospholipase D (PLD) has been identified as a potent exotoxin in *C. pseudotuberculosis*, and a key virulence factor in the development of CLA.

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

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

the lymphatic drainage of *C. pseudotuberculosis* in tissue fluid (Batey, 1986c).

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

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

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

from mycobacteria, the cytotoxic effect of *C. pseudotuberculosis* mycolic acid is confined to the site of injection (Hard, 1975).

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

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

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

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

Clinical and Pathological Features of Caseous Lymphadenitis in Sheep

C. pseudotuberculosis infections in sheep are classically associated with the formation of pyogranulomas (Valli and Parry, 1993), and this accounts for the name "caseous lymphadenitis". The lesions occur in two main forms, namely external (also known as superficial or cutaneous) and visceral, which may co-exist within the same animal. The external form is characterized by abscessation of those lymph nodes that may be palpated externally (Fig. 1). Any of the superficial lymph nodes of the body may be affected, dependent upon the original point of entry of the organism. Less commonly, localized purulent lesions not directly associated with the superficial lymph nodes may occur within the subcutaneous tissues. Such lesions may appear as organized abscesses, with swelling, fibrous encapsulation, loss of overlying hair and eventual



Fig. 1. A caseous lymphadenitis (CLA) abscess in the parotid lymph node of an adult ewe.

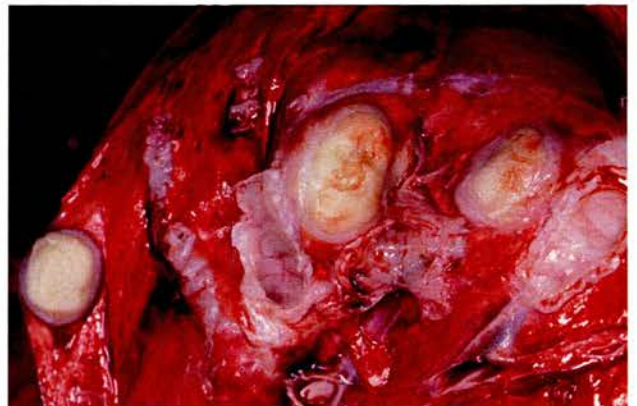


Fig. 2. CLA abscesses in the lung parenchyma. Note the close proximity of the lesion to the airways.

rupture, resulting in the discharge of pus (Radostits *et al.*, 2000).

The visceral form is associated with abscesses in the internal lymph nodes and other organs. In sheep, the principal location of these internal CLA lesions is the lung parenchyma and mediastinal lymph nodes (Fig. 2). Lesions may also be found in the liver, kidneys or udder, and more rarely the heart, testis, scrotum, uterus, joints, brain or spinal cord (Valli and Parry, 1993).

Pathogenesis in Sheep

There is a general consensus among recent reviewers regarding the stages through which *C. pseudotuberculosis* infection progresses. After initial entry, the organism spreads rapidly to the local drainage lymph node. Here, multiple microscopic pyogranulomas develop, growing in size and coalescing to form larger abscesses. This is sometimes followed by a further extension of infection via the blood or the lymphatic system, leading to similar lesions in other organs. The nature of these slowly developing CLA lesions means that chronic, and frequently lifelong, disease is the rule rather than the exception. Viable bacteria may be recovered from abscesses several years after initial infection. Reactivation of disease may also occur, with the development of lesions at new sites after a considerable period of apparent quiescence.

Numerous routes of inoculation have been used to induce experimental CLA in sheep; intradermal, subcutaneous, intravenous, intratracheal, intravaginal, and intralymphatic inoculation have all proved successful in establishing disease (Nagy, 1976; Burrell, 1978a; Pepin *et al.*, 1994b; Fontaine *et al.*, 2006). In natural infections, however, the principal route of entry is believed to be through the skin (Batey, 1986c; Brown and Olander, 1987; Davis, 1990; Collett *et al.*, 1994). This initial infection is facilitated by minor cutaneous wounds and abrasions, especially those caused by shearing (Paton *et al.*, 1988a). Wounds caused during castration or docking have also been suggested as an occasional route of entry, as has the umbilicus in neonatal animals (Valli and Parry, 1993). Entry via the oral cavity has been postulated to account for the small number of head and neck lesions seen in sheep from the Antipodes and North America, and the many more such lesions in goats (Ashfaq and Campbell, 1979). In contrast, the more distal parts of the intestinal tract are not believed to provide a portal of entry for the organism, even in the presence of parasitic damage (Valli and Parry, 1993).

A respiratory route of infection, postulated by Stoops *et al.* (1984), has been widely quoted in subsequent reviews. This theory was based on the observations that

some naturally infected sheep show only pulmonary lesions, and that a small number of these lesions are located within the walls of airways (Fig. 2). Moreover, Brown and Olander (1987) reported the production of disseminated pulmonary abscesses by injecting intratracheally a broth culture of *C. pseudotuberculosis*. However, other studies have indicated that such pulmonary lesions may develop as part of a systemic infection initiated elsewhere in the body. Thus, after the intravenous inoculation of lambs with *C. pseudotuberculosis*, the majority of internal lesions appeared in the lungs and associated thoracic lymph nodes (Brogden *et al.*, 1984). It has also been noted that in natural ovine CLA infections, the patterns of distribution of pulmonary lesions are consistent with haematogenous or lymphogenous spread rather than with aerogenous spread (Nairn and Robertson, 1974). It would therefore appear that entry of infection via the respiratory tract, although a theoretical risk, is of minor importance.

As already described, purulent cutaneous or subcutaneous lesions from which *C. pseudotuberculosis* may be isolated occur infrequently in sheep, even in flocks in which the incidence of classic lymph node lesions is high. It therefore follows that, on infecting the host via the skin, the organism is poorly suited to establishing persistent infections at the site of entry, and on most occasions must progress to the local drainage lymph node and beyond if infection is to be sustained (Valli and Parry, 1993). The means by which the organism spreads from the initial point of entry, eventually to form the classic lesion of CLA, has received close examination. Of crucial importance is the survival and replication of *C. pseudotuberculosis* in macrophages, which then carry the organism to the site of the eventual CLA lesion.

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

Once a lymph node has been colonized by *C. pseudotuberculosis* it undergoes a short period of generalized inflammation. PLD, the soluble exotoxin produced by *C. pseudotuberculosis*, is the probable initiator of this

lymphadenitis. Pepin *et al.* (1991) reported that within 24 h of the subcutaneous inoculation of lambs a number of micro-abscesses occurred within the cortical region of the lymph node draining the site of inoculation. By day six post-inoculation, these micro-abscesses had become more numerous and began to expand and coalesce to form larger purulent foci. The early pyogranulomas contained clumps of bacteria and cellular debris, with a relatively high proportion of eosinophils, giving the purulent core a slightly green hue. At the same time, and in parallel with the cellular events at the point of entry, the infiltration of neutrophils diminished and monocytes/macrophages became the predominant cell type within the lesion (Pepin *et al.*, 1994b). A process during which the lesion is encapsulated followed shortly thereafter, leading to a diminution of the inflammatory reaction in the parenchyma of the node. Continued slow expansion of the lesion sometimes then occurred, depending on the location of the node and whether or not it ruptured to discharge its contents. Such enlargement progressed through a repeated cycle of necrosis and reformation of the outer capsule. In the early stages, the purulent contents of the abscess were soft and semi-fluid; however, as time progressed, the pus within the lesion took on a more plastic or solid form, in which scattered clumps of bacteria were sometimes noted. Small nodules of mineralization formed within the purulent material, causing it to become progressively paler in colour. These calcified foci tended to be laid down in concentric layers reminiscent of the cross-sectional view of an onion (Figs 3–5). This classic “onion ring” presentation is regarded as virtually pathognomonic for CLA in countries such as Australia and New Zealand, but is rarely reported in the UK. This probably reflects the relatively small number of chronic infections amongst sheep in Britain, where *C. pseudotuberculosis* is a relatively recent arrival and infected animals are still likely to be culled after diagnosis (Baird, 2003). Superficial lymph node abscesses may expand to reach a diameter of as much as 15 cm, but 3–5 cm is a more common size (Valli and Parry, 1993).

The majority of reviews of ovine CLA have been written from a North American or Australian perspective. These reviews describe the external form of CLA as most commonly affecting the superficial lymph nodes of the torso (Nagy, 1971; Stoops *et al.*, 1984; Brown and Olander, 1987; Williamson, 2001). Ayers (1977), in a review of the disease in US sheep, reported that the precrural node was most commonly affected, followed by the prescapular and then by other superficial lymph nodes with equal frequency. The author regarded the occurrence of lesions in the head and neck as relatively rare in sheep, but more common in goats. A similar statement is made by an Australian author describing

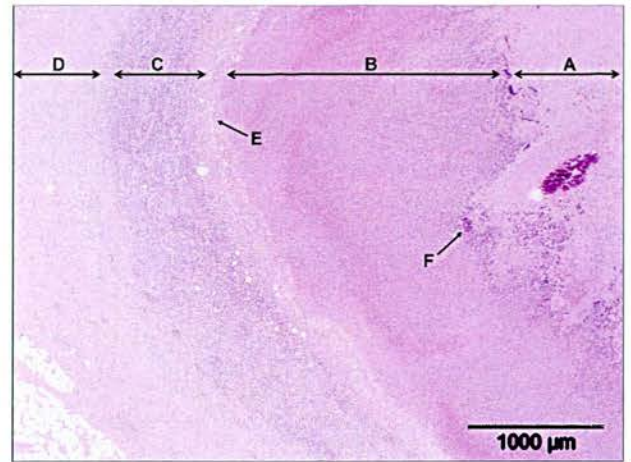


Fig. 3. Transverse histological section of a prescapular lymph node from a case of ovine caseous lymphadenitis. Several distinct concentric layers are discernible within the lesion. Centrally there is liquefactive necrosis (liquid pus; A) which is surrounded by coagulative necrosis (caseous pus; B), both containing multiple foci of mineralization (F), apparently in loosely arranged concentric layers. A thin layer of polymorphonuclear neutrophils surrounds the periphery of the coagulative necrosis; there is a further outer layer of coagulative necrosis containing polymorphonuclear neutrophils migrating through it at different densities, forming an apparent bi-layer (E). This is tightly bordered by a layer of immature fibrosis (C) containing mononuclear inflammatory cells. A thick layer of mature fibrosis (D) delineates the extent of the lesion. Haematoxylin and eosin (HE).

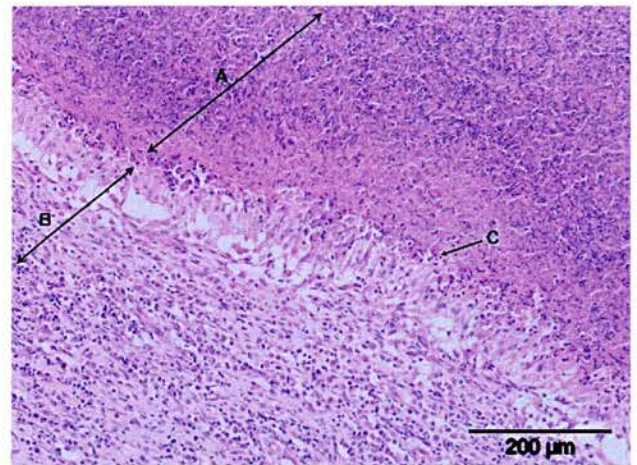


Fig. 4. Higher magnification of Fig. 3, showing the border between the layers of caseous necrosis (A) and active immature fibrosis containing mononuclear inflammatory cells (B). Note the presence of polymorphonuclear neutrophils migrating across the border into the caseous necrosis (C) and deeper. HE.

the usual presentation of the disease in that country (Batey, 1986b). Authors from New Zealand (Nuttall, 1988), South Africa (Collett *et al.*, 1994) and Canada (Muckle and Menzies, 1993) all describe a similar

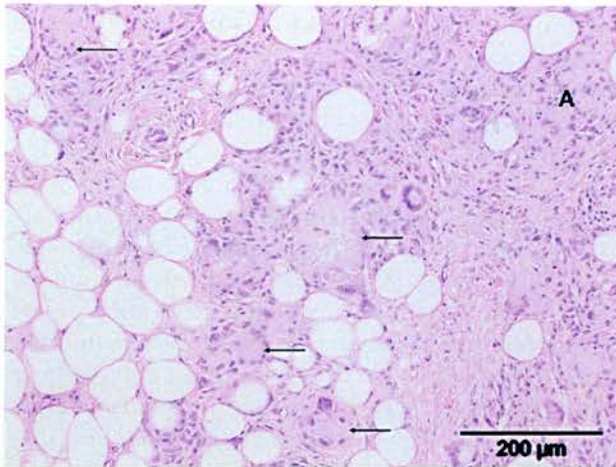


Fig 5. Transverse histological section of the interface between the outer edge of the mature fibrous capsule (A) and the surrounding tissue. Note the presence of many multinucleate giant cells (arrows). HE.

tendency for superficial lesions to develop particularly in the lymph nodes of the torso. This implies that, in the countries mentioned above, the skin of the torso is a frequent site of entry for the pathogen.

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

Other than the lymph nodes, the lung is the most frequent site of CLA lesions. As already noted, the generally random distribution of lesions within the parenchyma is consistent with haematogenous or lymphogenous spread, with seeded lesions developing from within the alveolar vasculature (Batey, 1986c). These pulmonary lesions most commonly take the form of encapsulated abscesses similar to those seen in the lymph nodes, but occasionally a more extensive bronchopneumonia is also recorded (Valli and Parry, 1993). The latter may lead to areas of pleuritis, resulting in the development of fibrinous or fibrous adhesions to the chest wall, pericardium or diaphragm (Renshaw *et al.*, 1979; Stoops *et al.*, 1984). In animals with pulmonary

abscesses, CLA lesions are occasionally encountered in the associated mediastinal and bronchial lymph nodes, implying an additional step in the transit of the organism from the lung parenchyma. Abscesses within the mediastinal lymph nodes may become so large as to put pressure on the adjacent oesophagus, interfering with normal swallowing and rumination, and leading to chronic ill-thrift (Paton *et al.*, 2005).

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

Economic Significance of Disease in Sheep

CLA is recognized as a significant cause of financial loss to the sheep industry in a number of countries where the disease is endemic. The major cause of these economic losses lies in the condemnation and downgrading of affected carcasses at slaughter and meat inspection. In Australia, where CLA-related losses have been extensively studied, it is estimated that the disease costs the meat industry \$A12–\$A15 million per annum (Paton *et al.*, 2003). This is due both to carcass losses and to the requirement for additional meat inspection and carcass trimming.

Systemic infection by *C. pseudotuberculosis* is acknowledged to be detrimental to the productivity of the infected animal, but to what extent is unclear. Studies of Merino sheep in Australia failed to show a relationship between carcass conformation and weight, and the occurrence or extent of CLA lesions (Batey, 1986b). However, workers in that country established that CLA infection had a detrimental effect on wool production. In a comparison between CLA-affected and unaffected Merino sheep in three Australian flocks, the loss of total clean wool production in the infected animals was assessed as 4.1–6.6% during the year of initial infection. Based on disease surveillance and wool production data from 1992, it was estimated that CLA infection cost the Australian sheep industry approximately \$A17 million per annum in lost wool production (Paton *et al.*, 1994). However, the same field workers were unable to confirm that CLA infection had any detrimental effects on the live weight gain of young sheep (Paton *et al.*, 1988b).

In contrast to much of the Southern Hemisphere, where the systemic effects of CLA on the individual sheep are regarded as marginal, in North America the disease is considered to be much more significant clinically. There, the visceral form of infection with *C. pseudotuberculosis* has been associated with so-called "thin-ewe syndrome", a chronic emaciation of ewes, occurring despite good appetite and in the absence of significant parasitic infestation or specific clinical signs. Two studies conducted in the US concluded that CLA infection had an economically significant effect on culling rates and reproductive efficiency in ewes (Gates *et al.*, 1977; Renshaw *et al.*, 1979), but Arsenault *et al.* (2003) considered that these studies lacked experimental controls. Certainly, the diagnostic criteria of "thin-ewe syndrome" are vague, and although *C. pseudotuberculosis* is the principal bacterial causative agent, other organisms such as species of *Moraxella*, *Arcanobacterium* and *Staphylococcus* may be present in mixed culture (Renshaw *et al.*, 1979). On occasion, *Moraxella* species may also be isolated in the absence of *C. pseudotuberculosis*. More significantly, there would appear to be some evidence of a synergistic relationship between CLA and maedi visna-induced pneumonia in sheep.

In the Middle East, considerable economic losses arise through the condemnation of lamb carcasses with CLA lesions, most commonly in the submandibular lymph nodes. Prevalence rates of 20–40% have been reported amongst lambs from intensively reared flocks of Najdi sheep (Pepin *et al.*, 1994a). CLA lesions in lambs destined for slaughter as part of religious festivals may render the carcasses virtually worthless—equivalent to a loss of \$200 per head.

Epidemiology of Infection in Sheep

Transmission of Infection

Much CLA research has focussed on how *C. pseudotuberculosis* cells, commonly contained within thick-walled abscesses, are transferred from one animal to another. Several possible routes have been suggested. The numbers of viable bacteria in purulent discharge from a CLA lesion have been estimated as between 10^6 and $5 \times 10^7/g$ (Brown and Olander, 1987). The rupture of superficial abscesses therefore releases huge numbers of viable bacteria on to the adjacent skin and fleece, and may readily contaminate the immediate environment. Other animals may then be exposed, either by direct physical contact with the affected animal, or indirectly via contaminated fomites. In addition, the ability of *C. pseudotuberculosis* to survive in the environment for a number of weeks or months extends the potential period of infection well beyond the point of initial rupture and discharge.

The recovery of organisms from the faeces of infected animals was recorded from the earliest days of CLA research (Seddon *et al.*, 1929) and its potential significance for disease transmission has been noted in subsequent reviews. However, in the absence of CLA lesions in the intestinal tract, the organisms probably originate from the respiratory tract, or represent bacteria that have traversed the gut, having been ingested. The fact that bacteria have also been isolated from the faeces of uninfected sheep would tend to support the latter hypothesis (Benham *et al.*, 1962). Despite the ability of *C. pseudotuberculosis* to survive in the environment for protracted periods of time, attempts to isolate the organism from the soil in areas of endemic infection have been unsuccessful (Knight, 1969). Therefore, the presence of *C. pseudotuberculosis* organisms in faeces and the consequent risk to other animals are likely to be of minor importance.

In CLA-infected sheep flocks, animals with lung lesions are thought to represent the principal source of infection for other animals (Pepin *et al.*, 1994a; Williamson, 2001). This conclusion is based principally on epidemiological observations recorded in Australia, in which the seroprevalence of CLA increased rapidly in groups of sheep, despite the absence of superficial CLA lesions (Ellis *et al.*, 1987; Paton *et al.*, 1988a). In addition, *C. pseudotuberculosis* has been isolated from the tracheae of sheep with pulmonary abscesses, confirming that discharge from lung lesions into the airways does indeed occur (Stoops *et al.*, 1984; Pepin *et al.*, 1994a). The assumption is made that even a relatively small number of animals with lung abscesses can create an aerosol of infectious organisms. Under conditions of close contact and reduced airflow, as in a covered shed, such an aerosol might be capable of infecting a large number of animals in a short period of time (Paton *et al.*, 1996).

The usual means of entry of a new infection into a naïve flock is via the introduction of a clinically or subclinically infected carrier animal (Ayers, 1977). A role for transmission of infection by farm workers and their equipment between animals within the same flock has been established. It has also been suggested that human activity may, on rare occasions, be responsible for introducing infection to previously disease-free flocks. In flocks with a high prevalence of CLA, shearers and their equipment are inevitably exposed to the purulent discharges of superficial CLA lesions. If subsequent decontamination measures are inadequate, it is possible that shearing gear, clothing and mobile handling equipment may act as a mechanical vector of infection to other flocks. A circumstantial link between new disease outbreaks in previously unaffected closed sheep flocks, and visits by contract shearers has been suggested in Western Australia, Canada and England; such reports, however, remain anecdotal.

Other mechanical vectors of infection have been suggested. The investigation of an outbreak of CLA in a closed and accredited disease-free goat herd in the Netherlands identified the most likely source of infection as bales of hay purchased from another farm. This second farm was known to have a herd of goats in which CLA was highly prevalent, and infected goats had been housed in the hay shed (Dercksen *et al.*, 1996).

Risk factors suggested for the spread of *C. pseudotuberculosis* infection amongst sheep include the breed (Bruere and West, 1990), increasing age of animals (Pepin *et al.*, 1994a; Paton *et al.*, 1995) and a dusty farm environment (Bruere and West, 1990). However, the principal risk factor identified in Australia and other parts of the world is minor skin damage due to shearing of the fleece (Nagy, 1976; Paton *et al.*, 1988a, 1996; Serikawa *et al.*, 1993; Al Rawashdeh and Al Qudah, 2000). In Australia, sheep are frequently housed or yarded together for a period immediately after shearing, before being returned to their grazings. This spell of close confinement is regarded as the period of greatest risk, during which the organism may pass via an infectious aerosol from one animal to another (Paton *et al.*, 1996). The risks inherent in shearing may be increased if a plunge or shower dipping is applied within a few days of shearing (Nairn and Robertson, 1974; Augustine and Renshaw, 1986; Ayers, 1986; Paton *et al.*, 1996), the suggestion being that organisms from affected animals persist in the dip solution for a period.

Comparison with Goat Infection

Natural CLA infections in goats demonstrate a number of similarities with the disease in sheep, not least that the same biotype of *C. pseudotuberculosis* is responsible. However, consideration of the differences in clinical presentation between sheep and goats may be instructive in respect of pathogenesis and specific risk factors. This is of particular interest in the UK, where the clinical presentation of ovine CLA is closely similar to that of the caprine infection in other parts of the world (Baird, 2003).

In both sheep and goats, the major sites of infection are the superficial lymph nodes, visceral lesions being encountered in only a minority of animals. In sheep, however, visceral lesions, and especially lung lesions, occur more frequently and in greater numbers than in goats (Renshaw *et al.*, 1979; Hein and Cargill, 1981; Ayers, 1986; Muckle and Menzies, 1993). In caprine CLA, the principal sites of superficial lesions are the nodes of the head and neck (Ayers, 1977; Burrell, 1981; Batey, 1986; Brown and Olander, 1987; Williamson, 2001). As already noted, most reports of ovine CLA indicate that head and neck lesions are uncommon, the more frequent site of abscessation being the superficial

lymph nodes of the torso. These observations are consistent with the notion that the main routes of infection in goats are via the oral cavity or via skin of the face and head. If bacteria are present on the skin or in the immediate environment, certain behavioural traits shown by goats, such as mutual grooming, head butting, and an inquisitive attitude (including browsing and mouthing behaviour), would increase the risk of exposure to infection. In contrast, such behavioural traits are less frequently observed in sheep. Most goats are neither shorn nor subjected to plunge or shower dipping.

Corynebacterium pseudotuberculosis Infections in Other Animal Species

C. pseudotuberculosis is pathogenic for a variety of mammalian species. Although a wide range of warm-blooded creatures are susceptible to the infection, specific disease syndromes are recognized in a smaller subset. In addition to the globally important diseases of sheep and goats, significant syndromes are recognized in horses and dairy cattle.

In horses, *C. pseudotuberculosis* is the cause of three recognized disease syndromes: ulcerative lymphangitis, contagious folliculitis and furunculosis, and deep-seated subcutaneous abscessation (Miers and Ley, 1980). In the US, *C. pseudotuberculosis* has also been recorded as a rare cause of abortion (Poonacha and Donahue, 1995) and mastitis in mares (Addo *et al.*, 1974), and with generalized visceral abscessation (Paton *et al.*, 2005). The three main equine syndromes tend to have a distinct geographical distribution, suggesting the importance of secondary disease factors. Indeed, the western areas of Canada and the US represent the only region in which all three syndromes have been recorded (Aleman *et al.*, 1996). In a study by Spier *et al.* (2004) a real-time polymerase chain reaction-based analysis of flies collected from the vicinity of horses with *C. pseudotuberculosis* infections detected the PLD-encoding gene in a significant proportion of test subjects; this was considered to indicate active carriage of *C. pseudotuberculosis*. In total, three fly species were identified as potential vectors for transmission of disease; significantly, *C. pseudotuberculosis* was identified in up to 20% of the houseflies (*Musca domestica*) examined.

In ulcerative lymphangitis, infection appears to enter via wounds, generally those of the lower limbs. This is followed by extension to the afferent lymphatic vessels and the formation of abscesses along their course. These abscesses appear as subcutaneous nodular lesions, which rupture to discharge pus and form necrotic ulcers (Radostits *et al.*, 2000). Such infections may persist for many months, as successive crops of ulcers develop and then resolve. Transmission commonly

occurs by direct contact between horses; an arthropod vector, however, has also been suggested to be of importance in Africa (Addo, 1983).

Equine folliculitis and furunculosis (also referred to as equine contagious acne, Canadian horsepox and equine contagious pustular dermatitis) is a much less clinically important form of disease, which probably represents a secondary infection by *C. pseudotuberculosis* of a pre-existing seborrhoea or dermatitis. The lesions tend to develop at points of contact with harnesses and other tack, and spread may occur through the use of contaminated grooming equipment. Again, the characteristic lesion is a small nodule that develops into a pustule before rupturing to discharge purulent material. These lesions usually resolve spontaneously but may occasionally result in an ulcer at the site of infection.

Deep-seated *C. pseudotuberculosis* abscessation (pigeon fever, Wyoming strangles, false strangles) is the most serious manifestation of the infection in horses. It is usually seen as abscesses in the musculature of the chest and shoulder, and is of economic significance in much of the western US (Hughes and Biberstein, 1959; Rumbaugh *et al.*, 1978; Miers and Ley, 1980), where its incidence is highest in the months of September to November (Aleman *et al.*, 1996; Doherr *et al.*, 1998). This probably reflects the seasonal incidence of various species of biting arthropod that act as mechanical vectors (Doherr *et al.*, 1999).

The recognized equine syndromes have not been described in the UK in recent years, despite the isolation of *C. pseudotuberculosis* from horses on a number of occasions (Connor *et al.*, 2000). The apparent significance of seasonality, environmental conditions and arthropod vectors in *C. pseudotuberculosis* infections of horses has been emphasized by a number of authors. It would therefore appear that, although equine pathogenic strains exist in the UK, essential contributory risk factors do not. It is noteworthy that ulcerative lymphangitis was not uncommon in the UK during World War I, but this probably resulted from the introduction of infected horses from Russia and the Balkans where the condition was present (Petrie and McClean, 1934; Benham *et al.*, 1962).

In recent years infections of dairy cattle have been recorded in Israel, commonly in the form of deep subcutaneous abscesses that develop into granulating and discharging ulcers, which respond poorly to systemic antibiotic treatment. The disease is sporadic but may sometimes occur in an epidemic form (Yeruham *et al.*, 2003b). Morbidity rates of up to 35% have been recorded in some Israeli dairy herds (Yeruham *et al.*, 1997). A less common mastitic form of disease is also recognized. This may be mild, the only sign being the appearance of clots in the milk, or severe, with a greatly enlarged and tender mammary gland and complete

cessation of milk production. The severe form often necessitates premature culling, as the response to antibiotic therapy is poor (Shpigel *et al.*, 1993). In a rare visceral form of disease, widespread abscessation occurs in lymph nodes associated with the upper and lower respiratory tract. In severe cases this may lead to swelling and obstruction of airways (Shpigel *et al.*, 1993). Finally, the organism has also been associated with a necrotic and ulcerative dermatitis of the heel in heifers, a morbidity rate of 91% having been reported in one outbreak (Yeruham *et al.*, 2003a). *C. pseudotuberculosis* has been recorded as a cause of an ulcerative lymphangitis and mastitis of cattle in countries other than Israel, albeit rarely (Purchase, 1944; Adekeye *et al.*, 1980; Kariuki and Poulton, 1982; Anderson *et al.*, 1990; Shpigel *et al.*, 1993).

C. pseudotuberculosis was isolated from houseflies collected over a *C. pseudotuberculosis* lesion in a cow (Yeruham *et al.*, 1996). Braverman *et al.* (1999) examined houseflies that had fed on a superficial *C. pseudotuberculosis* lesion in a cow, or on contaminated milk from animals with *C. pseudotuberculosis* mastitis. The study revealed that a significant proportion of flies harboured the organism either on their body surfaces or in their intestines, the bacterium being present in the faeces. It was concluded that this species of fly had a predilection for feeding on milk residues of cow teats, and therefore played an important role in harbouring and disseminating *C. pseudotuberculosis* infections in dairy herds in Israel.

C. pseudotuberculosis is commonly identified as the cause of a purulent lymphadenopathy affecting large numbers of farmed llamas and alpacas in Chile and other South American countries (Braga *et al.*, 2006). It is also encountered sporadically as the cause of abscesses in companion camelids in North America (Anderson *et al.*, 2004). In parts of the Middle East *C. pseudotuberculosis* has been isolated from lymph node abscesses of buffaloes (Ali and Zaitoun, 1999) and from similar lesions in camels (Esterabadi *et al.*, 1975; Nashed and Mahmoud, 1987; Abubakr *et al.*, 1999). Sporadic natural infections have been recorded in deer (Hammersland and Joneschild, 1937; Eghetti and McKenney, 1941), pigs (Zhao *et al.*, 1993), coypus, primates and ducks (Benham *et al.*, 1962) and a hedgehog (McAllister and Keahey, 1971). Experimental infections are readily induced in laboratory species such as mice, rabbits and guinea-pigs (Carne, 1940; Jolly, 1965).

Zoonotic Infections

Reports of human infection with *C. pseudotuberculosis* are relatively few, the first published case appearing some 40 years ago (Lopez *et al.*, 1966). Most human cases were classified as occupational infections, affecting

workers who had regular contact with sheep, such as shepherds, shearers, abattoir workers and butchers (House *et al.*, 1986; Peel *et al.*, 1997). Human infections tend to be chronic, presenting as a localized suppurative granulomatous lymphadenitis and affecting the axillary, inguinal or cervical lymph nodes (Mills *et al.*, 1997). The lymphadenitis may follow a period of influenza-like symptoms and increasing lethargy. Treatment with systemic antibiotics is generally unrewarding, the majority of cases requiring surgical excision of the affected lymph node. Even then, the healing of surgical wounds and infected sinuses may be protracted, and recurrence of lesions in other sites is not uncommon (Henderson, 1979). Because affected human lymph nodes are not always cultured after excision and because unspecified axillary lymphadenitis is not uncommon in shearers, it has been speculated that human *C. pseudotuberculosis* infections are under-reported in countries such as Australia, where ovine CLA is especially prevalent (Hamilton *et al.*, 1968). More serious human infections, extending beyond localised lymphadenopathy, have been recorded. On one occasion an eosinophilic pneumonia due to *C. pseudotuberculosis* was diagnosed in a veterinary surgeon from the US (Keslin *et al.*, 1979), but there are no published records of fatal human infections. A noteworthy case was recorded in a city-dweller, who had no apparent connection with a farming environment or livestock. In this instance, the only potential cause identified was the regular consumption by the patient of unpasteurized goats' milk (Goldberger *et al.*, 1981). No case of human infection with *C. pseudotuberculosis* has yet been reported in the UK; there is, however, a reference to an isolate of *C. pseudotuberculosis* from a human lymph gland submitted to the UK National Collection of Type Cultures (Hill *et al.*, 1978), but the origin of the sample was not mentioned.

A recent report described a case of *C. pseudotuberculosis* necrotizing lymphadenitis in a 12-year-old girl in France (Join-Lambert *et al.*, 2006), thought to be the first reported childhood case. The girl contracted the infection after having had contact with sheep while on vacation in a rural part of France. The nitrate-negative organism was susceptible *in vitro* to a range of common antibiotics, but treatment with amoxicillin and clavulanic acid failed to clear the infection. A second course of amoxicillin/clavulanate was then administered without success. After the infection had increased in severity and spread beyond the affected inguinal lymph node, intravenous antimicrobial therapy with imipenem/cilastatin, rifampicin and ofloxacin was administered, followed by surgical resection of affected tissue other than lymphatic tissue (to avoid post-lymphadenectomy oedema). Intravenous antibiotic administration was continued for a further 4 months, followed by

oral rifampicin and ofloxacin treatment for a further 6 months. Two years after the cessation of treatment, no relapse had occurred.

Control and Eradication of Disease in Sheep

Diagnosis

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

In the past, identification of *Corynebacterium* spp. has proved difficult for several reasons. These reasons include (1) insufficient numbers of reference strains with which to compare isolates, (2) inadequacies in the reference data obtained from characterized strains, (3) the relative lack of appropriate criteria, and (4) the inappropriate use of test criteria, since coryneform bacteria frequently give rise to negative results with orthodox tests (Hill *et al.*, 1978). It has long been considered that *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans* are related, as they share a similar morphology and cell wall composition, and all produce a characteristic halo on Tinsdale medium, indicating the production of a cystinase enzyme (Barksdale, 1970). However, on the basis of DNA homology, there would seem to be less similarity between the three species than was at one time thought (Groman *et al.*, 1984). Nonetheless, *C. pseudotuberculosis* and *C. ulcerans* can be made to produce diphtheria toxin by infecting them with toxin-associated phages from *C. diphtheriae* (Petrie and McLean, 1934; Saxholm, 1951; Henriksen and Grelland, 1952; Maximescu, 1968; Maximescu *et al.*, 1974a,b).

In the laboratory, *C. pseudotuberculosis* cultured from clinical samples may be identified from its enzymatic profile and its ability to utilize various carbohydrate sources. The task of biochemical profiling has been greatly simplified by the introduction of standardized and miniaturized proprietary test kits, an example of which is the Analytical Profile Index (API)

identification system (bioMérieux [UK], Basingstoke, Hampshire, UK). The "API Coryne" kit (used for the identification of coryneform bacteria) comprises 21 individual test substrates for the determination of enzymatic activity or carbohydrate fermentation. After inoculation with the test organism and incubation for a defined period, the metabolic end-products of the enzymatic tests result in the development of specific colour changes, either spontaneously or following the addition of reagents. For substrate fermentation tests, pH change is also detected colorimetrically. Subsequently, a particular combination of positive and negative test results is used to compile a numerical profile (an Analytical Profile Index; API), which is then used to "interrogate" a proprietary software database.

For the API tests, computer software is used to calculate the percentage fit of the test isolate results to a consensus profile for the identified species. The database takes into account natural intra-species variation in certain biochemical attributes. The use of computers to aid in bacterial identification (so-called "probabilistic" identification) was reported as far back as 1973 (Bascomb *et al.*, 1973; Lapage *et al.*, 1973; Willcox *et al.*, 1973). Briefly, the methodological process described at the time was to record the results of different taxonomic (e.g., biochemical) tests as the probability of a positive result (ranging from 0.99 to 0.01). The results obtained for the test isolate were then recorded as either "+" or "-", and a computer programme was used to compare these test results with the probabilities associated with the corresponding tests for each bacterial taxon in the database. Subsequently, the probabilities were used to calculate an 'identification score' for the test isolate, which was then compared with the identification scores for those taxa in the database. The final outcome was the identification of the test isolate, complete with the probability of a correct identification. Subsequently, an identical technique was applied to the identification of coryneform bacteria, and impressive results were obtained, including the successful use of the database to identify field isolates of *C. pseudotuberculosis* (Hill *et al.*, 1978).

In addition to substrate utilization, further laboratory-based tests are available to identify *C. pseudotuberculosis*. Some years ago, it was noted that when β -lysin-producing staphylococci were cultured on blood-containing solid media in the presence of strains of *Streptococcus agalactiae* that produce a secreted 'factor', enhanced zones of haemolysis were observed. This led to the discovery of an *S. agalactiae* protein, designated CAMP-factor (after the initials of its discoverers, Christie, Atkins and Munch-Petersen), which on its own was unable to cause erythrocyte lysis. The synergistic lysis phenomenon was designated the CAMP-reaction (Christie *et al.*, 1944). Similarly, when *C.*

pseudotuberculosis and *Rhodococcus equi* are cultured together on solid blood-containing media, colonies of both species in close proximity to each other cause significant zones of erythrocyte lysis (Fraser, 1961). A similar phenomenon was noted when *C. pseudotuberculosis* was cultured in the presence of δ -lysin-producing staphylococci (Lovell and Zaki, 1966). The *R. equi* protein (so-called equi factor) involved in synergistic lysis was found to be a phospholipase C enzyme (Bernheimer *et al.*, 1980). Currently this assay, which may also be performed by supplementing blood-containing solid media with *R. equi* culture supernate (containing equi factor), is used in diagnostic laboratories for the identification of *C. pseudotuberculosis* (Fig. 6). However, it should be noted that *C. ulcerans* also produces PLD, although significantly it is the only other corynebacterial species found to do so (Barksdale *et al.*, 1981). Hence, PLD activity is a distinctive marker within the genus *Corynebacterium*.

In contrast to the synergistic lysis described above, it was reported (Soucek *et al.*, 1962) that *C. pseudotuberculosis* exerted an inhibitory effect on the haemolysis produced by staphylococcal β -lysin, and the inhibitory agent was thought to be associated with PLD. A comparable inhibition of haemolytic activity was also exerted upon the α -toxin of *Clostridium perfringens* (Soucek *et al.*, 1967). As with the synergistic lysis test, the so-called reverse-CAMP, or CAMP-inhibition test (Fig. 7) is still exploited to this day in diagnostic laboratories, although the same issue of the production of PLD by *C. ulcerans* must again be taken into account.

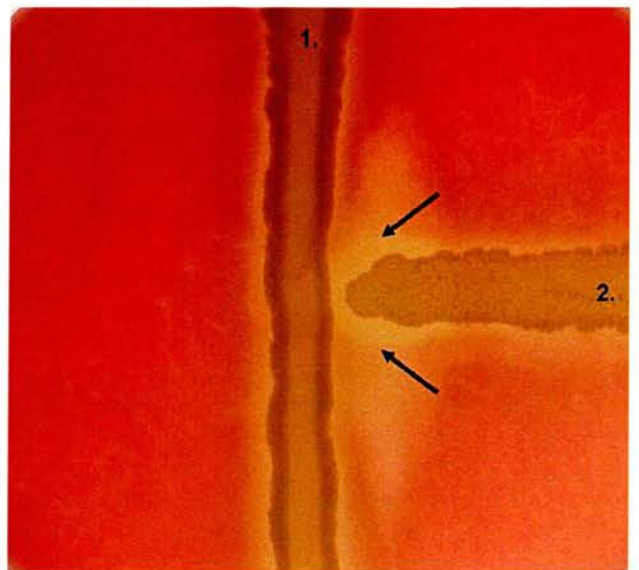


Fig. 6. Synergistic lysis between *Rhodococcus equi* (1) and *Corynebacterium pseudotuberculosis* (2). The zone of enhanced lysis (arrows) is apparent where the soluble factors from each organism come into contact with each other.

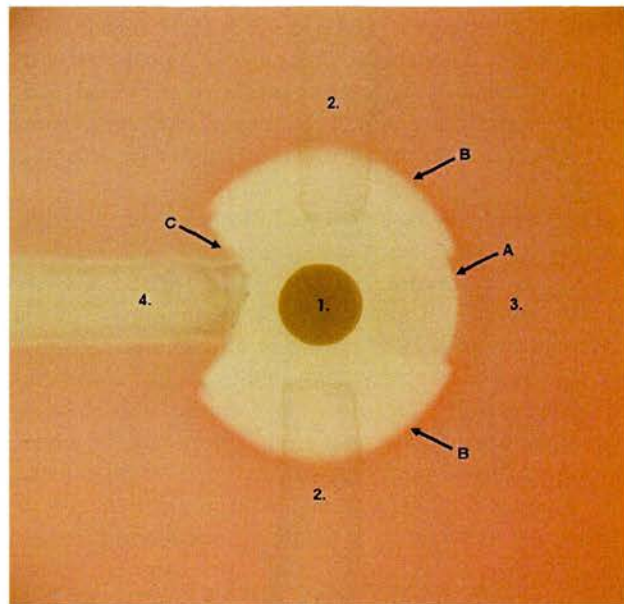


Fig. 7. The CAMP-inhibition test. Erythrocyte lysis resulting from diffusion of *Staphylococcus aureus* (1.) haemolysin is apparent (A), as also is enhanced lysis resulting from the synergistic effects of *Streptococcus agalactiae* (2.) CAMP factor and *S. aureus* haemolysin (CAMP reaction; B). The PLD-deficient control strain, *Corynebacterium jeikeium* (3.), has no effect on the extent of the CAMP reaction, while diffusion of PLD by *C. pseudotuberculosis* (4.) is inhibitory (C).

Treatment

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

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

Serology

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

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

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

Other tests used in the study of CLA include tube agglutination assays (Cameron *et al.*, 1972; Husband and Watson, 1977), an indirect haemagglutination test (Shigidi, 1978) and a double immunodiffusion test (Burrell, 1980b). However, the enzyme-linked immunosorbent assay (ELISA) for use in diagnosis has shown particular promise. Initially CLA-diagnostic ELISAs employed crude *C. pseudotuberculosis* cell wall preparations or supernate-derived exotoxin as antigen (Maki *et al.*, 1985; Sutherland *et al.*, 1987; Kuria and Holstad, 1989; Ellis *et al.*, 1990; Sting *et al.*, 1998). Test sensitivity was usually regarded as good, but specificity was relatively poor—possibly due to cross-reactions with other proteins present in the antigen preparations. However, some ELISAs have been claimed to be sufficiently specific and sensitive for field diagnosis. One such ELISA was based on a PLD antigen derived by recombinant technology from *E. coli* containing a plasmid bearing the *pld* gene (Menzies *et al.*, 1994), a test sensitivity of 86.3% and specificity of 82.1% being reported. The assay coming closest to the requirements of a diagnostic test was reported by workers in the Netherlands (Ter Laak *et al.*, 1992) and was later refined to improve specificity and sensitivity (Dercksen *et al.*, 2000). This is an indirect double antibody sandwich ELISA, based on PLD exotoxin purified from the supernate of *C. pseudotuberculosis* culture, polyclonal rabbit anti-PLD serum being used as a capture antibody. The improved method is reported to have a specificity in sheep of $99 \pm 1\%$, and a sensitivity of $79 \pm 5\%$.

Other potential tests include a polymerase chain reaction (PCR) method (Cetinkaya *et al.*, 2002) and a bovine interferon (IFN)- γ whole blood ELISA (Menzies *et al.*, 2004). The latter was the first test in which assay of cellular immunity to *C. pseudotuberculosis* was used as a diagnostic tool. Over the course of 1 year, the IFN- γ test accurately detected goats experimentally infected with *C. pseudotuberculosis* with a reliability of 89.2%, and non-infected goats with a reliability of 97.1%. Over the same period, a recombinant PLD ELISA detected the experimentally infected goats with a reliability of 81.0% and non-infected goats with a reliability of 97.0%. The PLD ELISA was, however, more predictive than the IFN- γ ELISA of the lesions subsequently

observed at necropsy. A later study of INF- γ production in cultures of whole blood compared infected goats following stimulation with either secreted bacterial antigen or somatic antigen; IFN- γ production by blood cells was found to be significantly higher in response to the secreted antigen (Meyer *et al.*, 2005).

The use of serology as a tool in disease eradication and control has been pioneered in the Netherlands, principally in relation to dairy goats. Indeed, a CLA Health Scheme for goats exists in that country, based upon the use of a PLD antibody ELISA and Western blot analysis (Dercksen *et al.*, 1996). A similar scheme was proposed for the control and eradication of CLA in Dutch sheep (Schreuder *et al.*, 1994), but the plans foundered due to poor test sensitivity in certain flocks (Dercksen *et al.*, 2000). Plans for serological control of the disease in sheep have recently been revived in the UK, with the launch in 2005 of a pre-sale testing regime incorporating the use of a serodiagnostic ELISA and Western blot analysis based on a recombinant PLD antigen (Anon, 2005).

Vaccination

Historical perspective. Despite the relative lack of knowledge pertaining to *C. pseudotuberculosis* virulence factors, there exists a significant body of work exploring the potential for immunization to prevent disease caused by this organism. Due to the relatively recent introduction of CLA into the UK, there has been little research originating from this country; however, the same is not true of elsewhere in the world. In particular, significant work has been conducted in both South Africa and Australia over the last 75 years. Many of the earliest reports were somewhat confusing, partly because lack of knowledge at the time prevented isolation of the organism in a pure form and characterization of the cellular antigens. As stated by Batey (1986c), "it is likely that infection by *C. pseudotuberculosis* involves a unique mode of pathogenesis"; these words continue to bear weight as progress towards an understanding of this organism is advanced through a combination of molecular dissection and an accurate analysis of the interaction of pathogen with host.

One of the earliest reports, cited by Petrie and McClean (1934), of protection against the deleterious effects of *C. pseudotuberculosis* products appeared in 1907, when a degree of resistance against the lethal effect of the exotoxin (PLD) was apparently produced in guinea-pigs by administration of a high dose of horse-derived antiserum against the toxin of *C. diphtheriae* (Petrie and McClean, 1934). This work was performed at a time when it was thought that there was a relationship between the exotoxins of *C. pseudotuberculosis* and *C. diphtheriae*. It was subsequently shown that the two

toxins were distinct, and that the protective effect observed at the time was probably due to the prior occurrence of anti-*C. pseudotuberculosis* antibodies in the horse from which the antitoxin was derived (Petrie and McClean, 1934). Later work revealed that immunization with toxoided *C. pseudotuberculosis* exotoxin gave some protection to guinea-pigs against the lethal effects of the active exotoxin (Petrie and McClean, 1934). Despite this observation, the general consensus of opinion held that the exotoxin was unsuitable for use as a protective antigen against CLA in sheep (Carne *et al.*, 1956; Cameron and Smit, 1970) due to the chronic nature of the natural disease, in contrast to the acute nature of *C. pseudotuberculosis* infection in mice and guinea-pigs.

In the early 1960s it was suggested that the immunizing property of *C. pseudotuberculosis* was associated with the toxicity of cell endoplasm preparations and with the ability of formalin-killed cells to produce sterile abscesses when injected into guinea-pigs (Cameron, 1964). However, it was subsequently shown that these 'toxic factors' and those responsible for conferring immunity to infection were in fact distinct (Cameron and Buchan, 1966). Later studies showed that mice could be significantly protected against challenge with *C. pseudotuberculosis* by immunization with formalin-killed, washed, whole *C. pseudotuberculosis* cells, combined with one of a variety of adjuvants (Cameron and Minnaar, 1969). The cellular component believed to confer the most immunity was the cell wall. Through a process of elimination it was discovered that the antigen responsible was an "integral part of the cell wall", but was not the cell wall lipid, which did not appear to induce protective immunity (Cameron *et al.*, 1969). The ability of cell wall components to induce immunity was reduced by prior treatment with formalin; the reason for this was thought to be the denaturation of cell wall-associated protein or proteins, resulting in a loss of biological activity, which was believed to be required for immunogenicity (Cameron *et al.*, 1969).

In continuing previous work, Cameron and Smit (1970) investigated toxic components in *C. pseudotuberculosis* protoplasmic preparations, with a view to understanding the cause of the sterile abscesses formed on inoculation of animals with killed *C. pseudotuberculosis* cells. It became apparent that a toxic cytoplasmic component was identical with the exotoxin found in culture supernates. However, consistent with an earlier report (Petrie and McClean, 1934), Cameron and Smit hypothesized that this extracellular exotoxin must have arisen through lysis of bacterial cells, since the concept of protein secretion was still not understood. Nonetheless, their work clarified to some extent the confusion surrounding the various toxic fractions studied in previous years.

In the following section the different approaches to vaccine development will be discussed. However, it is not within the scope of this review to discuss the merits of any given approach or the acceptability of the resulting vaccines from a commercial or regulatory standpoint.

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

More recently, in a study of several vaccines, immunization of sheep with a formalin-killed, virulent, UK-derived *C. pseudotuberculosis* isolate and aluminium hydroxide adjuvant resulted in statistically significant protection against homologous challenge; the vaccine had prevented the spread of infection beyond the site of inoculation (Fontaine *et al.*, 2006).

There have been few investigations of the protective effect of *C. pseudotuberculosis* bacterins against disease in the field. Menzies *et al.* (1991) conducted a field trial of a killed whole-cell vaccine in a flock of sheep and herd of goats, both populations having shown a prevalence of CLA in adult animals ranging from 15% to 30% in the pre-trial period. During the trial, lambs and kids were vaccinated at 2.5–3.5 months of age, with two further doses administered at 1 month and 11 months after the initial vaccination. The animals were regularly monitored for 36 months after the initial dose of vaccine. Serum antibody concentrations were significantly increased in vaccinated sheep and goats for the full term of the experiment. Furthermore, vaccination significantly decreased the incidence of CLA in the sheep, and there was a suggestion of a similar effect in the goats.

In a later study, juvenile sheep and goats were vaccinated with a *C. pseudotuberculosis* bacterin formulated

in light mineral oil containing muramyl dipeptide (Brogden *et al.*, 1995); the results, however, were inconclusive.

Toxoid vaccines. As noted above, Petrie and McClean (1934) found that vaccination of guinea-pigs with a formalin-inactivated exotoxin (PLD) of *C. pseudotuberculosis* induced a degree of protection against homologous challenge. Despite this, it was generally considered that the chronic nature of *C. pseudotuberculosis* infection in sheep rendered toxoid ineffective as a protective antigen. Most subsequent studies on CLA vaccination focussed on the use of bacterins, or on vaccines made from components of whole cells. However, a report by Jolly (1965) showed that the administration of toxoid, followed by toxin, reduced the extent of experimental CLA in sheep. This followed the observation by Cameron (1964) that an undefined "protoplasmic toxin" of *C. pseudotuberculosis* played a role in the induction of protective immunity. It was later demonstrated that *C. pseudotuberculosis* exotoxin was in reality identical with this protoplasmic toxin (Cameron and Smit, 1970), adding weight to the hypothesis that PLD could be used as a protective antigen.

Purely managerial strategies having failed to prevent the spread of the disease, studies were conducted in Australia during the 1970s to develop a CLA vaccine, work that would eventually lead to the first commercial CLA vaccine (Eggleton *et al.*, 1991a,c). The ability of PLD to protect against CLA was reported by both Nairn *et al.* (1977) and Burrell (1978b). Work at the Division of Animal Health, Commonwealth Scientific and Industrial Research Organisation (CSIRO), led to the optimization of procedures for the culture of *C. pseudotuberculosis*. It also resulted in improvements in the yields and quantification methods of PLD, matters of importance in view of its immunogenic properties (Burrell, 1983).

Between 1977 and 1978, the technology required for production of an effective toxoid vaccine was made available to the Commonwealth Serum Laboratories (CSL; Parkville, Victoria, Australia), and details, including optimal antigen dose and combination with other (clostridial) components, were later published (Eggleton *et al.*, 1991c). For vaccine production, PLD was obtained from filtered and concentrated *C. pseudotuberculosis* culture supernates, which were subsequently toxoided with formalin (Burrell, 1983; Hodgson *et al.*, 1999). A combined clostridial and corynebacterial vaccine, known as GlanvacTM, was released in 1983 (Eggleton *et al.*, 1991a); it subsequently became available in Australia and several other countries but is not licensed for use in the European Union. In one report, vaccination of goats with GlanvacTM resulted in statistically significant protection against subsequent experimental infection with *C. pseudotuberculosis*, as measured

by a reduction in the number of lesions (Brown *et al.*, 1986). In addition, the results of trials performed in the process of the commercial development of GlanvacTM indicated significant protection of sheep against CLA (Burrell, 1983; Eggleton *et al.*, 1991a-c). Anderson and Nairn (1984), however, found that PLD toxoid gave greater protection when not combined with clostridial components.

The mechanism by which GlanvacTM confers immunity has been the topic of some debate. It has been suggested that protection against CLA is both humoral and cell-mediated, but that PLD is neutralized by circulating antibody (Burrell, 1978b; Batey, 1986c; Eggleton *et al.*, 1991a; Sutherland *et al.*, 1992). In contrast, Hodgson *et al.* (1999) discussed the possibility that the small amount (*ca* 1%) of PLD activity that remained after formalin treatment (Eggleton *et al.*, 1991b) might be responsible for the protection conferred by GlanvacTM. However, Eggleton *et al.* (1991b) had previously reported that a four-fold variation in the level of active toxin remaining in a toxoid vaccine "neither enhanced nor reduced the protective potency of the vaccines". Others have reported that anti-exotoxin antibodies are not on their own sufficient to prevent infection in the absence of other (probably cell-mediated) immune defences (Irwin and Knight, 1975; Cameron and Bester, 1984; Ellis *et al.*, 1990). Significantly, Cameron and Smit (1970) stated that "...it could be expected that crude exotoxin preparations could also contain other antigens. Therefore, unless an absolutely pure preparation of exotoxin can be produced, its immunizing activity can never be accurately assessed". This statement was later reiterated by Burrell (1983), who confirmed that the culture supernates used to prepare GlanvacTM contained other *C. pseudotuberculosis* somatic antigens, which varied in amount from batch to batch. Moreover, Walker *et al.* (1994) intermittently identified antibodies against a novel *C. pseudotuberculosis* antigen in sera from sheep vaccinated with GlanvacTM. It would therefore seem that the presence of other *C. pseudotuberculosis* proteins in toxoid vaccines prepared from culture supernates may well contribute to the protective capacity of the vaccine.

Fontaine *et al.* (2006) assessed the capacity of a highly purified recombinant derivative of PLD to protect sheep against homologous challenge with a UK-derived *C. pseudotuberculosis* isolate. Protection was similar to that produced by a bacterin vaccine. After experimental challenges, infection appeared to be localized to the inoculation site in all but one experimental animal, in which evidence of pulmonary infection was also found. Given that PLD does not stimulate cell-mediated immunity (Hodgson *et al.*, 1999), the protection observed in animals vaccinated with the recombinant PLD was postulated to result from antibody-mediated

neutralization of toxin activity. Included in the same study was an assessment of the capacity of the PLD-based proprietary vaccine, Glanvac-3TM (a member of the GlanvacTM vaccine family), to protect against challenge with the UK-derived *C. pseudotuberculosis* isolate. Statistically significant protection was again observed with this vaccine, as indicated by a decreased number of infected loci. GlanvacTM vaccine appeared to be less successful than the recombinant PLD vaccine in preventing the spread of the challenge infection beyond the site of inoculation and into the superficial lymph nodes; it was conceded, however, that the immunity produced by the GlanvacTM vaccine was demonstrated by challenge with a heterologous bacterial strain. However, the high degree of sequence conservation between the PLD-encoding genes from different *C. pseudotuberculosis* isolates may well negate the issue of homologous versus heterologous challenge.

Combined vaccines. In reviewing CLA vaccine development, Burrell (1983) stated that, despite the capacity of cell-associated antigens (Cameron *et al.*, 1969) and PLD (Burrell, 1978b) to give some protection, full protection required the presence of both. In defence of this, it was revealed that an alhydrogel-adjuvanted vaccine composed of formalin-killed whole *C. pseudotuberculosis* cells, enriched with formalin-treated, PLD-rich culture supernate, protected completely against experimental *C. pseudotuberculosis* infection and outperformed vaccines consisting of cell-free toxoided culture supernates (Burrell, 1983). In contrast, a report of work conducted during the development of GlanvacTM revealed that supplementation of cell-free toxoid vaccine with *C. pseudotuberculosis* cells made no difference to the observed protection (Eggleton *et al.*, 1991b).

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

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

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

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

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

It was later postulated (Hodgson *et al.*, 1994) that the rationale behind the creation of the Toxminus mutant was flawed: while the recombinant strain was attenuated for virulence and hence useful as a live vaccine vector, one of the major immunodominant antigens (PLD) had been removed, thus reducing immune stimulation. Hodgson *et al.* (1994) therefore constructed a mutant derivative of PLD in which the histidine₂₀ amino-acid residue (a constituent of the enzyme's active site) was substituted for tyrosine, and expression of the resulting plasmid-borne, mutant *pld* gene produced a biologically inactive derivative of PLD. On transformation of Toxminus with the inactivated *pld* construct, cells were able to secrete an inactive PLD derivative, albeit at a level lower than that of wild-type PLD.

In an attempt to avoid inoculation-site reactions and to provide a convenient means of vaccine administration,

Hodgson *et al.* (1994) administered each of two vaccines (Toxminus and Toxminus carrying the inactivated *pld*) to sheep via the oral route. Both vaccines induced significant and similar humoral immune responses. Furthermore, in animals immunized with the strain expressing the inactivated *pld*, challenge elicited a rapid anamnestic response to PLD; this response was quicker and of greater magnitude than the humoral response (1) to infection in unvaccinated animals, or (2) in those receiving Toxminus alone. Despite significant humoral responses, the protection against challenge conferred by the Toxminus strain was negligible; this contrasted with a previous report of subcutaneous vaccination with Toxminus (Hodgson *et al.*, 1992). The difference in protection was attributed to the failure of oral vaccination, unlike subcutaneous vaccination, to stimulate a significant Th1 immune response, IgG1 having been identified as the dominant IgG isotype. Despite the failure of Toxminus alone, immunization with Toxminus expressing the mutant *pld* gene resulted in significant protection against challenge, confirming the earlier observations that antibodies against PLD protected against CLA (Jolly, 1965; Nairn *et al.*, 1977; Burrell, 1978a; Eggleton *et al.*, 1991b, c).

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

Other reports of attempts to create live vaccine vectors against CLA have been generally less successful than those discussed above. Disruption of the *aroQ* gene, encoding 3-dehydroquinase (an essential enzyme in aromatic amino-acid biosynthesis), led to the creation of attenuated mutants of two strains of *C. pseudotuberculosis*, one possessing and one lacking PLD. Both strains were cleared from the liver and spleen of mice within 8 days of intraperitoneal inoculation (Simmons *et al.*, 1997). In contrast, complementation of the chromosomal *aroQ* mutation, by introduction of a plasmid

encoding the wild-type *aroQ* gene, was sufficient to restore the virulence of the mutant strains to some extent, but not to the level shown by the wild-type parent strain. Protection studies with one of the attenuated mutants revealed that high doses induced a degree of protection against wild-type challenge, in addition to the stimulation of INF- γ production by murine splenocytes. INF- γ plays a role in macrophage activation, and the suppression of *C. pseudotuberculosis* growth and lesion development is largely due to the bactericidal action of activated macrophages (Hard, 1970). Therefore, the vaccine potential of the attenuated strains was evident; it was observed, however, that both were effectively cleared from INF- γ -deficient mice, suggesting that the attenuation allowed clearance by some other, undefined route. This, in turn, was considered useful, given that vaccination would be unlikely to cause infection, even in immunocompromised animals (Simmons *et al.*, 1997).

Despite the encouraging initial results in mice, trials in sheep proved disappointing in respect of the protection against CLA offered by the AroQ mutants (Simmons *et al.*, 1998). Consistent with previous results, both mutants showed reduced ability to persist within lymph nodes and to produce local reactions at the inoculation site. However, both failed to stimulate detectable production of specific INF- γ -secreting lymphocytes and induced only low concentrations of anti-*C. pseudotuberculosis* IgG. Not surprisingly, these immune responses did not protect sheep from infection.

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

In an effort to improve the efficacy of DNA vaccination against intracellular pathogens, fusion of antigen-encoding genes to those encoding specific "effector" molecules has been attempted. One such effector molecule is the cytotoxic T lymphocyte antigen-4 (CTLA-4) (Brunet *et al.*, 1987), which assists in directing secreted vaccine antigens to lymph nodes and antigen-presenting cells (APCs) (Boyle *et al.*, 1998; Drew *et al.*, 2001). CTLA-4, a repressor of T-lymphocyte proliferation, is expressed on the surface of activated T cells. In its dimeric form it competes with CD28 for binding to the CD80 and CD86 (B7 domains) on antigen-presenting cells (Linsley *et al.*, 1991; Thompson and Allison, 1997). A truncated derivative of CTLA-4 (designated CTLA-4-Hlg)

was created, composed of the C-terminal end of the extracellular domain fused to the hinge CH2 and CH3 domains of human IgG1 (HIg); this derivative was able to dimerize as a result of the IgG hinge domains. Subsequently, chimeric proteins, consisting of antigen-encoding sequences fused to CTLA-4-HIg, were found to be specifically targeted to APCs (Boyle *et al.*, 1998). In a later study it was shown, in vaccinated mice, that the antibody response to a *T. ovis* 45W antigen-CTLA-4-HIg fusion construct was 30 times higher than that achieved with non-targeted DNA vaccination. Furthermore, the kinetics of the antibody response to the targeted construct were faster than achieved with non-targeted DNA or with an adjuvanted protein vaccine; unfortunately, vaccination of outbred sheep with the fusion vaccine failed to enhance immune responses (Drew *et al.*, 2001).

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

Identification of immunodominant antigens. In attempts to develop more refined CLA vaccines, PLD has been the main focus of research. This was due, in part, to the perceived importance of the toxin in the dissemination of *C. pseudotuberculosis* within the infected host (Carne and Onon, 1978) and to the observation that anti-PLD antibodies assist in clearance of *C. pseudotuberculosis* from

experimentally infected animals (Eggleton *et al.*, 1991a). It is also probable that, in the absence of a *C. pseudotuberculosis* genome sequence and of information on other virulence determinants, PLD was favoured for inclusion in novel vaccines. However, as discussed above, *C. pseudotuberculosis* cell-associated antigens also contribute to protection against CLA (Brogden *et al.*, 1990; Hodgson *et al.*, 1992), and have even been suggested to be essential for the induction of optimal immunity (Cameron *et al.*, 1969; Cameron, 1972; Burrell, 1983).

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

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

The results obtained through use of ASC probes were significant, since they revealed that the CP40 antigen, which was found in all *C. pseudotuberculosis* strains examined, was one of the earliest products to be recognized

by the host during infection (Walker *et al.*, 1994). There was no obvious relation between CP40 antibody titres and the amount of antigen administered or incidence of lung lesions, implying that cell-mediated immunity was the main protective mechanism.

Caseous Lymphadenitis in the United Kingdom

Although it is generally accepted that CLA is a relatively new arrival to the UK, there exists a tantalizing report by Heath and Batty (1952) concerning the isolation of an "unusual diphtheroid" bacterium from three Swaledale sheep from the same flock in southwest Cumberland. Based on colony and cell morphology and biochemical tests, these isolates corresponded almost exactly to a previous description by Morse (1949) of *C. pseudotuberculosis* (at that time *C. ovis*). Realizing the potential implications, Heath and Batty consulted two other authorities. One considered that the organism, although a member of the genus *Corynebacterium*, was not *C. pseudotuberculosis* because it was non-pathogenic for guinea-pigs, did not "attack" urea and probably contained metachromatic granules. The other observed that the organism was not virulent for sheep but otherwise resembled *C. pseudotuberculosis*. In concluding their report, Heath and Batty (1952) stated that the organism might be a different variety of the same species (i.e., *C. pseudotuberculosis*) and that it was worthy of record in view of the extremely rare isolation of *Corynebacteria* (other than *C. pyogenes*) from British sheep.

Given the inconclusive nature of the report referred to above, and the absence of any further literature pertaining to *C. pseudotuberculosis* infections in the UK, the currently accepted first cases of infection in live animals in this country are those confirmed in January 1990 by workers at Cambridge University (Lloyd *et al.*, 1990). The diagnosis was made in two dairy goats from a farm in the south of England, these animals being part of a group of 20 Boer goats originally imported from the then West Germany in 1987 (Robins, 1991). *C. pseudotuberculosis* infection, although not a notifiable disease at that time, was still of interest to the UK State Veterinary Service. The farm on which the affected goats were kept was therefore placed under immediate movement restrictions and the tracing of prior movements of the batch of imported goats led to the confirmation of CLA in herds on a further five premises. The goats on each of these premises were either slaughtered or placed under movement restrictions. In addition, a group of in-contact sheep was slaughtered, but the animals showed no lesions of CLA at necropsy.

In June 1991, superficial abscesses were noted in a number of sheep during a routine examination of a flock in Dorset. Bacteriological investigations led to

confirmation of *C. pseudotuberculosis* infection. Once again sheep movements related to the affected flock were traced and as a result the infection was confirmed in a further seven sheep flocks. During the subsequent investigations no link to recently imported animals could be established. The affected flocks, found in various parts of southern England, were composed of purely indigenous animals. Consequently, after considering the extent of infection and the fact that CLA was present within most countries of the European Community, the UK Government decided that no further action would be taken in relation to the disease in either sheep or goats. All existing movement restrictions on affected goat herds and sheep flocks were therefore lifted (Robins, 1991).

In the immediate aftermath of these events, diagnoses of CLA in both sheep and goats as recorded on the national Veterinary Investigation Diagnosis Analysis (VIDA) database remained in low single figures (Fig. 8). The first case of ovine CLA in Scotland was reported in the Roxburghshire area of the Borders (Anon, 1996) and this coincided with increased publicity for the disease in the veterinary and farming press. According to VIDA statistics, the incidence subsequently rose sharply, reaching a peak of 62 diagnoses in 1998 and a further peak of 85 diagnoses in 2005 (VIDA, 1995, 2003, 2006, 2007). Cases of ovine CLA were also reported in Northern Ireland (Malone *et al.*, 2006) and in the Irish Republic (O'Doherty *et al.*, 2000), affecting animals imported from Scotland and England, respectively.

During the late 1990s, concerns were expressed over the potential under-reporting of new outbreaks in the UK (Baird, 1997; Rizvi *et al.*, 1997; Smith *et al.*, 1997; Winter, 1997; Binns *et al.*, 2002). The disease was often stated to be a particular problem of flocks from the "terminal sire breeds". In addition, there was a perception that infection was particularly prevalent amongst the rams in these flocks. Reports from England and Scotland identified outbreaks in which up to 60% of adult males showed clinical signs of CLA, while females showed a much lower prevalence (Scott *et al.*, 1997; Watson and Preece, 2001; Anon, 2006). Speculation on the reasons for this difference has focussed on particular management systems applied to breeding males.

The risks to sheep farming presented by a high CLA prevalence in rams of the terminal sire breeds have been identified by a number of authors (Watson and Preece, 2001; Binns *et al.*, 2002; Baird, 2003; Baird *et al.*, 2004). More recently, the disease has been diagnosed in breeds associated with hill and upland settings, several outbreaks being reported in rams from North Country Cheviot flocks in the north of Scotland (Anon, 2004).

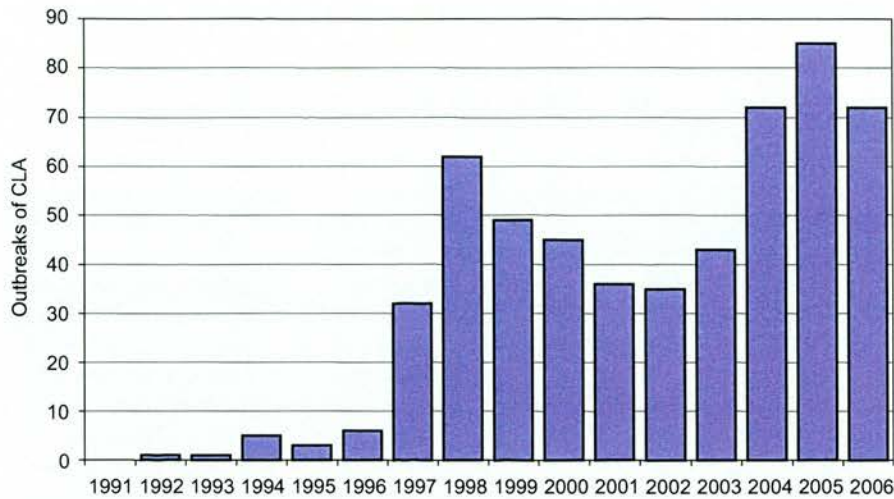


Fig. 8. Recorded outbreaks of CLA in GB sheep flocks from 1991 to 2006: Source: Veterinary Investigation Diagnosis Analysis (VIDA) database.

Future Direction

Debate continues over the best methods of controlling CLA in the UK. Many in the farming community advocate the licensing of a proprietary vaccine. Vaccination is the main strategy in many other countries where CLA is endemic. However, recent research would suggest that simply licensing pre-existing proprietary vaccines will not provide the solution that many in the farming community might expect (Fontaine *et al.*, 2006). Other vaccines may in the future provide enhanced protection, but much further work is required.

It is the authors' opinion that accurate serodiagnosis, together with segregation or culling of infected animals, offers a method for the complete eradication of CLA. In the absence of either this approach or the use of vaccination, there would seem little to prevent the eventual spread of CLA to all parts of the sheep industry in the UK, providing an object lesson in how a disease may spread from a point source to an endemic state. Improvements in serodiagnosis and vaccination will require continued effort to clarify the mechanisms by which *C. pseudotuberculosis* interacts with its host, causes disease, and often persists throughout life.

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Survey of caseous lymphadenitis seroprevalence in British terminal sire sheep breeds

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CASEOUS lymphadenitis (CLA) is a chronic, suppurative disease caused by infection with the bacterium *Corynebacterium pseudotuberculosis*, which affects several animal species but is rare in human beings (Peel and others 1997). The disease is common in sheep and goats in many parts of the world (Pepin and others 1994), but was believed to be absent from Great Britain until 1990 (Lloyd and others 1990). Examination of laboratory data indicates that outbreaks in British sheep flocks reached a peak in 1998, before falling in recent years (VIDA 2002). However, such data are subject to several important sources of bias and there is a need to establish more robust estimates of the prevalence of CLA infection in Great Britain.

There is currently concern that CLA is most common within the terminal sire breeds, suggesting a potential for further spread within the wider sheep industry. Experience in Britain indicates that a disproportionate number of rams are

affected by the disease (Baird 2000, Binns and others 2002), possibly as a result of management practices which favour the spread of infection among adult males. Rams are also a frequent vector of disease between otherwise closed flocks (Watson and Preece 2001). A study of CLA prevalence within rams of the terminal sire breeds is therefore a logical starting point in any consideration of the current disease situation within Great Britain.

Sheep flocks that have membership of the Maedi-Visna Accreditation Scheme, one of the sheep and goat health schemes operated by the Scottish Agricultural College (SAC), are blood tested biennially to confirm freedom from maedi-visna virus infection. At each flock test all active rams over 18 months of age must be blood sampled. Since the majority of the 3000 flocks within the accreditation scheme come from the terminal sire sector, a serological survey of samples from member flocks provides a cross-section of that part of the British sheep industry.

A double antibody sandwich ELISA has been developed to detect antibodies to the *C pseudotuberculosis* exotoxin, phospholipase D. The calculated diagnostic specificity of this ELISA in sheep is 99±1 per cent and the sensitivity is 79±5 per cent (Dercksen and others 2000), making it well suited for use as a tool in disease prevalence studies.

For a period of nine months during 2000, serum samples were collected from flocks within the sheep and goat health schemes. A total of 2538 samples from rams in 745 flocks were then tested blind using the ELISA, with results expressed as net extinction values. Values below 100 were considered negative, values of 100 to 200 as inconclusive and values above 200 as positive. In cases in which an inconclusive result was obtained by ELISA, further Western blot analysis was performed, with six clear, visible bands being taken as a positive result.

In total, 244 sera gave positive ELISA net extinction values and a further six samples were positive by Western blot. This

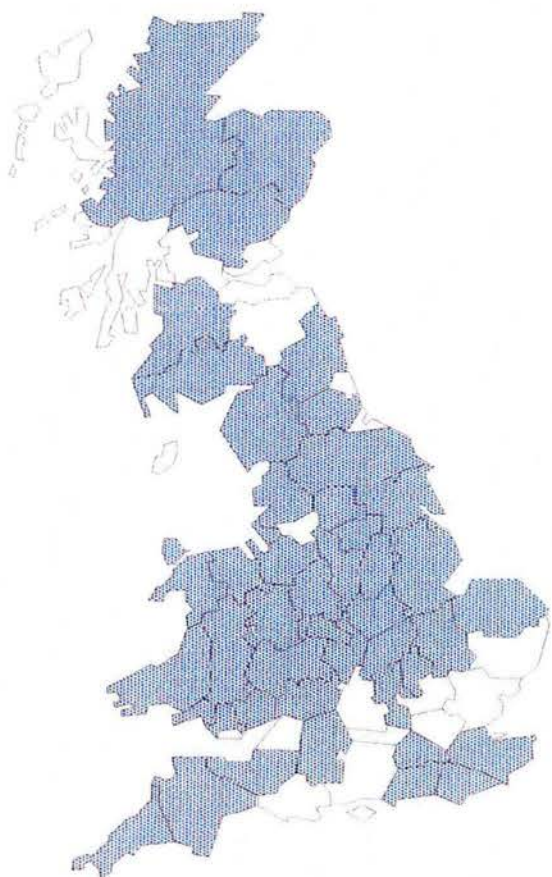


FIG 1: Results of a survey in 2000 showing regions or counties of Great Britain with sheep flocks containing rams seropositive for caseous lymphadenitis (blue shading)

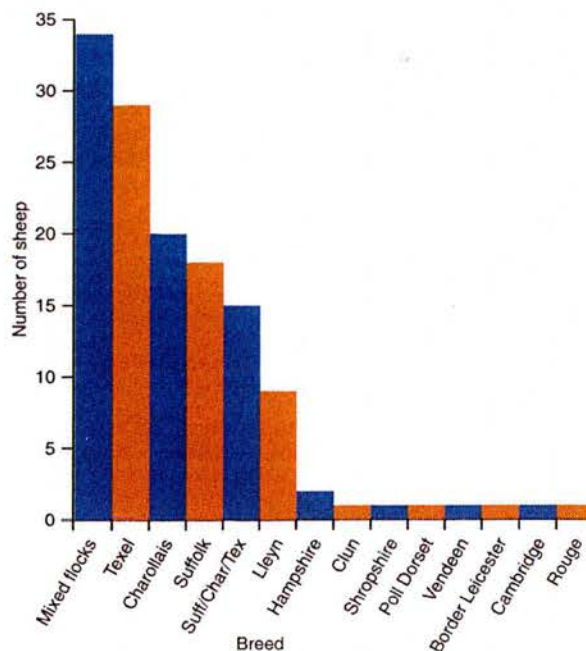


FIG 2: Recorded breed of flocks containing caseous lymphadenitis antibody-positive rams from a serological survey in 2000 (total 134). Flocks within the 'mixed breed' category are composed of sheep from two or more pure breeds. Those flocks in the 'Suff/Char/Tex' category are composed of sheep from two or more of the Texel, Charollais and Suffolk breeds only

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gave an overall apparent prevalence of seropositive animals within the sample group of 9.93 per cent (95 per cent confidence limits 8.76 per cent and 11.1 per cent, 99 per cent confidence limits 8.39 per cent and 11.4 per cent). Eighteen per cent of the total flocks sampled (134 of 745 flocks) had one or more ELISA-positive animals; 48 (36 per cent) of these had more than one positive animal, while on 10 occasions five or more positive animals were detected within the same flock.

While acknowledging the limitations inherent in the sampling method, this study suggests that CLA is well established within the main terminal sire breeds in Great Britain and that the infection is present in most parts of the country (Fig 1). In fact, the rather low test sensitivity recorded for the ELISA suggests that the true flock seroprevalence may actually be higher than the 18 per cent calculated here. Flocks from a wide range of sheep breeds appear to be affected by the disease (Fig 2); however, the temptation to over-interpret the data should be avoided. For example, it is noted that more than 60 per cent of the seropositive flocks are composed of sheep from the Texel, Charollais and Suffolk breeds. This finding has less significance than might at first appear, as these breeds account for a similar percentage of the Maedi-Visna Accreditation Scheme membership.

The findings of this study are broadly in line with the small number of published reports of UK outbreaks and with anecdotal evidence from veterinary practitioners, farmers and contract shearers. The results are of significance in terms of disease control and provide the basis for a larger-scale prevalence study, within both the terminal sire sector and the wider sheep industry in Great Britain.

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Escherichia coli O116 associated with an outbreak of calf diarrhoea

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ENTEROHAEMORRHAGIC *Escherichia coli* (EHEC) are a group of verocytotoxin-producing *E coli* (VTEC) that cause bloody diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome and thrombocytopenic purpura in human beings. The pathogenicity of these bacteria is mainly mediated by shiga toxins (Stx1, Stx2 and their variants) and the products of the locus of enterocyte effacement (LEE). The LEE contains the *eaeA* gene encoding intimin, a bacterial outer membrane protein involved in the intimate adhesion of bacteria to enterocytes and the production of characteristic attaching and effacing lesions (Paton and Paton 2002). In addition, the EHEC harbour a large plasmid, which carries a marker gene, *hlyA*, encoding a haemolysin termed enterohaemolysin (Schmidt and others 1995).

The first reports of Stx production by *E coli* strains isolated from calves with diarrhoea were published by Kashiwazaki and others (1980) and Wilson and Bettelheim (1980). The association between diarrhoea in calves and VTEC was later confirmed by Hall and others (1985) and Mohammad and others (1985), and several other studies suggested that the strains which produce Stx1 only and possess the *eae* gene were more involved in calf diarrhoea (Mainil and others 1993, Orden and others 1998). Stx-producing *E coli* have been associated with diarrhoea in one- to eight-week-old calves, with a peak between four and five weeks of age, although newborn and older calves have also been affected (China and others 1998). Similarly, VTEC have been detected in calves with diarrhoea under 16 weeks of age in Germany (Wieler and others 1996) and less than three months of age in Spain (Blanco and others 1996). In India, however, there have been no reports of VTEC in animals with diarrhoea and few reports of the isolation and molecular characterisation of VTEC from human beings with bloody and watery diarrhoea (Gupta and others 1992, Khan and others 2002a). Chattopadhyay and others (2001) were first to report the isolation of VTEC from non-diarrhoeic animal sources in India. Similarly, Khan and others (2002b), while investigating the prevalence and genetic profiling of non-O157 VTEC in Calcutta, detected *stx1* and *stx2* in 18 per cent of normal cow stool samples. To the authors' knowledge, this is the first report of an association of EHEC O116 with an outbreak of diarrhoea in calves.

An outbreak of bloody diarrhoea occurred in one- to 16-week-old crossbred calves in an organised dairy farm in Kashmir, India. The outbreak began in the third week of July 2002 and continued for one week. Seven of 10 calves were affected, presenting with diarrhoea for four to five days and voiding blood-tinged, liquid, malodorous faeces. Other signs included lethargy and dehydration. All the affected calves recovered. Faecal samples from the affected calves were screened for rotavirus by a sandwich ELISA and RNA-polyacrylamide gel electrophoresis (PAGE), followed by silver staining (Wani and others 2004), and were screened for salmonella and *E coli* as described by Wani and Gupta (1986) and Wani and others (2004). The *E coli* isolates were serogrouped by the National Salmonella and *Escherichia* Centre, Kasauli, India.

E coli strains were subjected to multiplex PCR for the detection of *stx1*, *stx2*, *eaeA* and EHEC *hlyA* genes. To extract bacterial DNA, each *E coli* strain was grown overnight in nutrient

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Papers

Control of caseous lymphadenitis in six sheep flocks using clinical examination and regular ELISA testing

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In an effort to control the spread of caseous lymphadenitis (CLA) infection, flocks of affected sheep on six holdings were tested serologically at regular intervals using an antibody ELISA with a mean (sd) specificity of 99 (1) per cent and a sensitivity of 79 (5) per cent. Western blot assays to detect antibodies to the phospholipase D (PLD) exotoxin of *Corynebacterium pseudotuberculosis* were used as a further test when ELISA results were inconclusive. Owners were advised to remove from the flock any sheep that demonstrated clinical signs of CLA or tested positive for PLD by ELISA or western blot. Of the six trial flocks, one was dispersed after only two blood tests, and in another the recommendations for CLA control were not followed and infected animals were retained within the flock. In the remaining four flocks, the testing regimen and other advice enabled the disease to be controlled to such an extent that the appearance of new clinical cases of CLA was effectively halted. This remained the case for up to five years after the end of the trial. In two of these flocks, a small number of seropositive animals were detected at the last flock test). However, on the other two holdings all sheep were seronegative in the final two flock tests, consistent with the complete eradication of infection.

CASEOUS lymphadenitis (CLA) of sheep, which is caused by *Corynebacterium pseudotuberculosis*, has been a significant disease in much of the sheep-rearing world for more than a century. The chronic and often subclinical nature of the infection makes the condition difficult to control (Baird and Fontaine 2007). As a consequence, disease prevalence has remained high in many regions, leading to significant economic losses associated with poor production and carcass condemnations at slaughter and meat inspection (Pepin and others 1994, Williamson 2001).

C. pseudotuberculosis was first introduced into the UK in the late 1980s (Robins 1991), and since then the infection has spread widely within the sheep and goat populations (Binns and others 2002, Baird 2003, Baird and others 2004, Baird and Fontaine 2007). In the UK, CLA infections most often cause abscessation of the superficial lymph nodes of the head and neck; the lymph nodes of the limbs and torso are less commonly affected. These lesions are generally recognised as firm, discrete swellings beneath the skin. The visceral form of the disease is also commonly found in the UK. In one study of infected flocks in Northern Ireland, lesions were present in the lungs of one-third of CLA-

affected sheep (Malone and others 2006). In the same study, internal CLA lesions only (involving the lungs and/or internal lymph nodes) were present in almost one-quarter of CLA-affected sheep.

In vitro, *C. pseudotuberculosis* is sensitive to a range of antibiotics (Muckle and Gyles 1982, Judson and Songer 1991). However, in vivo, clinical CLA is generally refractory to antibiotic therapy. This resistance is believed to be due to the intracellular nature of the organism during much of the process of pathogenesis and to the fibrous wall and thick, dry contents of the CLA abscess (Williamson 2001). Surgical treatment of external lesions and prolonged antibiotic treatment have been suggested as alternatives to culling in cases in particularly valuable sheep (Davis 1990); however, the long-term effectiveness of surgical intervention (Gezon and others 1991) and antibiotic treatment (Rizvi and others 1997) is generally poor.

CLA remains a common disease of sheep and goats in many parts of the world. In countries where proprietary vaccines are widely available, a significant degree of control can be established in infected flocks through planned and regular vaccination. Even under these circumstances, although disease prevalence may be greatly reduced, total eradication of the condition is not achieved.

Although the production of autogenous vaccines against CLA is allowed in the UK, proprietary vaccines, which are used to control CLA in other parts of the world, are not licensed for use. Moreover, recent research suggests that simply licensing pre-existing proprietary vaccines for use in the UK might not provide the complete solution that advocates of this approach expect (Fontaine and others 2006). Novel vaccines may provide enhanced protection in the future, but much further work is required. Thus, other methods of controlling and eradicating the disease must be considered seriously.

To date, control of the disease has relied on the physical examination of newly purchased sheep before their introduction into naive flocks and the culling of clinically affected sheep from flocks in which CLA is known to be present. However, the high level of

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TABLE 1: Results of serial serological examinations for caseous lymphadenitis in six sheep flocks

Flock	ELISA result	Round									Total number of seropositive sheep	
		1	2	3	4	5	6	7	8	9		
A	Number (%) positive	4 (5.3)	1 (1.6)									5
	Number (%) negative	71 (94.7)	61 (98.4)									
	Number (%) inconclusive	0	0									
	Total sheep	75	62									
B	Number (%) positive	17 (10.8)	7 (5.3)	4 (4.2)	1 (0.9)	1 (1.0)	0	0				30
	Number (%) negative	135 (85.4)	113 (86.3)	89 (93.7)	99 (88.4)	86 (89.6)	94 (100)	62 (100)				
	Number (%) inconclusive	6 (3.8)	11 (8.4)	2 (2.1)	12 (10.7)	9 (9.4)	0	0				
	Total sheep	158	131	95	112	96	94	62				
C	Number (%) positive	68 (63.0)	12 (34.3)	5 (8.5)	18 (37.5)	22 (44.0)	19 (26.4)	15 (21.4)	0	0		159
	Number (%) negative	30 (27.8)	23 (65.7)	54 (91.5)	29 (60.4)	27 (54.0)	52 (72.2)	55 (78.6)	94 (98.9)	41 (100)		
	Number (%) inconclusive	10 (9.3)	0	0	1 (2.1)	1 (2.0)	1 (1.4)	0	1 (1.1)	0		
	Total sheep	108	35	59	48	50	72	70	95	41		
D	Number (%) positive	17 (28.8)	9 (17.6)	16 (35.6)	14 (66.7)	13 (59.1)						56
	Number (%) negative	39 (66.1)	42 (82.4)	23 (51.1)	4 (19.0)	9 (40.9)						
	Number (%) inconclusive	3 (5.1)	0	6 (13.3)	3 (14.3)	0						
	Total sheep	59	51	45	21	22						
E	Number (%) positive	5 (7.8)	1 (1.7)	2 (2.9)	0	2 (2.9)*						10
	Number (%) negative	59 (92.2)	51 (89.5)	63 (92.6)	80 (98.8)	62 (91.2)						
	Number (%) inconclusive	0	5 (8.8)	3 (4.4)	1 (1.2)	4 (5.9)						
	Total sheep	64	57	68	81	68						
F	Number (%) positive	0	2 (9.1)	0	0	2 (9.1)*						4
	Number (%) negative	21 (91.3)	19 (86.4)	23 (100)	22 (100)	19 (86.4)						
	Number (%) inconclusive	2 (8.7)	1 (4.5)	0	0	1 (4.5)						
	Total sheep	23	22	23	22	22						

* No lesions detected on examination of animals after culling

subclinical infection means that efforts to detect carrier sheep and to remove sheep showing external signs of disease cannot totally control the disease.

Essential to the bacterium's ability to establish itself and spread through the body is the phospholipase D (PLD) exotoxin, a sphingomyelin-specific enzyme that catalyses the dissociation of sphingomyelin into ceramide phosphate and choline (Bernheimer and others 1980). Although a number of significant biological activities have been attributed to PLD, the most important in terms of pathogenesis is the damage that it causes to mammalian cell membranes (Pepin and others 1994). This activity allows *C. pseudotuberculosis* to resist destruction within phagocytic cells (Yozwiak and Songer 1993) and promotes carriage of the bacterium from the original site of infection within local tissue fluids (Batey 1986).

The role of PLD in ovine and caprine infections has made it the principal component of the proprietary CLA vaccines available around the world, and the detection of antibodies to PLD is the basis of specific serological tests for CLA. These assays have been explored as a means of controlling the disease in sheep through the identification and removal of infected carrier sheep. Most promising in recent years has been the development of an ELISA for diagnosis of the disease.

Initially, the antigens used in CLA-diagnostic ELISAs were preparations of *C. pseudotuberculosis* cell wall or exotoxin derived from culture supernatants (Maki and others 1985, Sutherland and others 1987, Kuria and Holstad 1989, Ellis and others 1990, Sting and others 1998). In the majority of these assays, the test sensitivity was good, but specificity was relatively poor as a result of cross-reactions with extraneous protein molecules within the antigen preparations. The developers of more recent ELISAs have claimed specificity and sensitivity levels sufficient to allow their use as diagnostic tools in the field. Improvements include the use of purer sources of PLD antigen, such as that derived by recombinant technology from *Escherichia coli* containing a plasmid bearing the *pld* gene (Menzies and others 1994). Menzies and others (1994) reported a test sensitivity of 86.3 per cent and a specificity of 82.1 per cent. Another ELISA method has been reported to have a test specificity of 100 per cent, a sensitivity of detection of total antibody of 71 per cent (95 per cent confidence interval [CI] 63 to 78 per cent), and a sensitivity of detection of IgG antibody to *C. pseudotuberculosis* of 83 per cent (95 per cent CI 76 to 89 per cent) (Binns and others 2007). The sensitivity of the IgG antibody assay was believed to be higher because of the greater affinity of IgG class antibodies compared with the IgM antibodies also detected by the total antibody ELISA.

The ELISA that comes closest to the requirements of a diagnostic test was developed in the Netherlands (ter Laak and others 1992) and was later refined to improve specificity and sensitivity (Dercksen and others 2000). This assay is an indirect double antibody sandwich ELISA that uses PLD exotoxin purified from the supernatant of *C. pseudotuberculosis* cultures and polyclonal rabbit anti-PLD serum as a capture antibody. The improved method is calculated to have a specificity of mean (sd) 99 (1) per cent and a sensitivity of 79 (5) per cent in sheep. The high specificity of this ELISA and moderate sensitivity make the test suitable for a test-and-cull scheme.

The ELISA method originally described by ter Laak and others (1992) and improved by Dercksen and others (2000) has been used in an established disease eradication and control scheme in the Dutch dairy goat industry. Dercksen and others (2000) reported a specificity of 98 (2) per cent and sensitivity of 94 (3) per cent for the improved ELISA in goats. A scheme for the eradication of CLA and subsequent accreditation of freedom from disease has been available to goat producers in the Netherlands for a number of years (Dercksen and others 1996). By 1996, 46 herds and 11,000 goats had been accredited, and trade in stock was then established between members. By 2007, the membership of the scheme had risen to 605 herds, representing approximately 220,000 accredited goats.

A similar health scheme using the earlier version of the ELISA test was proposed for the control and eradication of CLA in the smaller Dutch sheep industry (Schreuder and others 1994), although problems of test sensitivity in some flocks led to its closure (Dercksen and others 2000). The test was subsequently modified and the sensitivity in sheep was improved by Dercksen and others (2000). However, no further attempts have been made to establish a CLA health scheme in what is a relatively small Dutch industry.

Materials and methods

Sheep and management

Sheep from six flocks were clinically examined and samples of blood were taken for testing using an ELISA for CLA at regular intervals over a four-year period. Three of the flocks were located in Scotland, one was in England and two were in Northern Ireland.

Farm A was a mixed enterprise on approximately 22 ha farming a small herd of pedigree Charolais cattle and a flock of pedigree Suffolk sheep flock (flock A). At the start of the study, the trial flock was approximately 50 breeding ewes, and its management was closed apart from the periodic purchase or hiring of rams. A laboratory diagnosis of CLA was made for the first time in February 1997.

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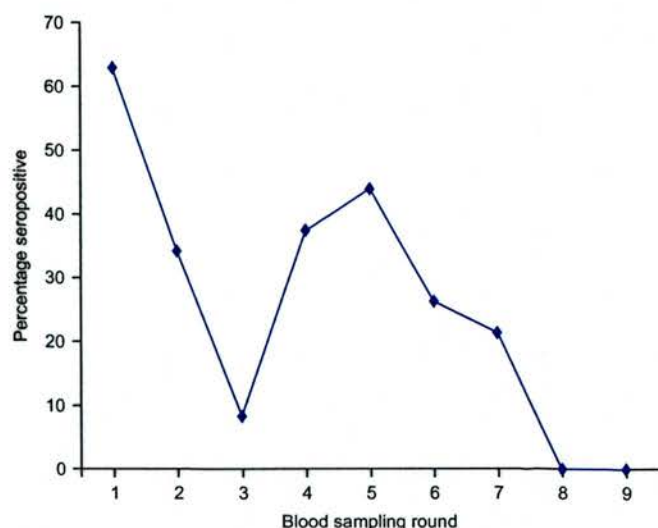


FIG 1: Seroprevalence of caseous lymphadenitis in flock C during the eradication trial

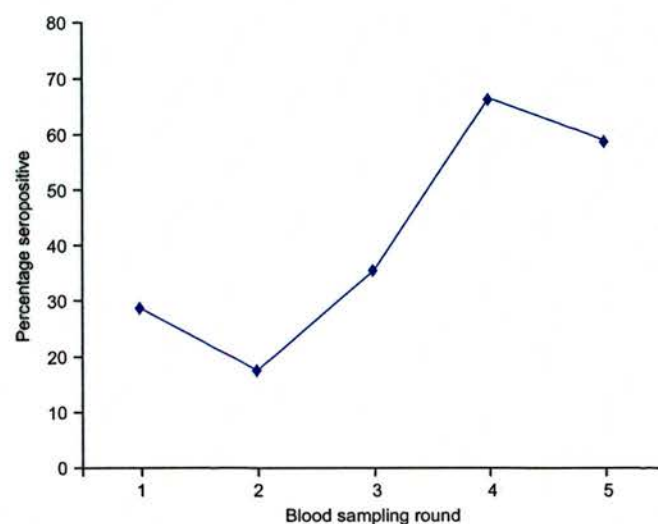


FIG 2: Seroprevalence of caseous lymphadenitis in flock D during the eradication trial

The source of the CLA infection was considered to be a shearing ram purchased during the autumn of 1996. After a period in the trial, towards the end of 1999, the flock owner decided to cease active farming. A dispersal sale of the entire Suffolk sheep flock took place shortly after.

Farm B had a suckler cattle herd, a commercial sheep flock and a pedigree Suffolk sheep flock (flock B), the latter supplying rams for home use and for sale. A diagnosis of CLA was made within the Suffolk flock for the first time in March 1997. The source of the CLA infection was believed to be a shearing ram that had been leased for a period of six months to flock A. On its return to flock B, this ram and other in-contact rams developed clinical signs of CLA, and the diagnosis was confirmed by cultures. In the year 2000, stock numbers on the farm were reduced, with a flock of approximately 60 breeding Suffolk ewes being retained.

Farm C had a pedigree Suffolk flock of approximately 75 breeding ewes (flock C) and a small livery stable for horses and ponies on approximately 10 ha. CLA was diagnosed for the first time within this flock in 1999. During the process of establishing the Suffolk flock, breeding sheep had been purchased from a number of sources, and it is believed that this is how the infection entered the farm. After the initial bacteriological diagnosis of CLA infection, the flock was managed under essentially closed conditions.

Farm D had a small pedigree charollais sheep flock (flock D) of approximately 20 breeding ewes, as part of a much larger farming enterprise. The flock produced rams for use in the larger commercial flock, and any additional sheep were sold for breeding. The first diagnosis of CLA was made in 1997, and the infection was believed to have entered the flock through breeding stock purchased within the previous few years.

Farm E had a flock of approximately 60 breeding pedigree Suffolk ewes (flock E) on a smallholding with no other livestock. The first diagnosis of CLA was made in January 1999. The affected sheep had been purchased in November 1998.

Farm F had a charollais sheep flock of 25 breeding ewes (flock F). Although this flock was on the same holding, it was managed at separate premises from a commercial flock of approximately 190 cross-bred ewes. The first cases of CLA were diagnosed in July 1999 and involved two shearing rams, both of which had been born on the farm. A further five cases were confirmed in rams between August and November 1999. It was believed that CLA had entered the flock with sheep purchased during the previous two years.

At the start of the study, the flock owners were given specific advice on management changes likely to reduce the spread of CLA infection within their flocks. This advice included the immediate isolation of suspected clinical cases pending bacteriological confirmation by removing the suspect animals from the flock and keeping them in an area where nose-to-nose contact was not possible. Treatment of housing and pens that had held sheep known to be infected, by means of steam cleaning and use of a disinfectant solution from the Defra-approved list (Defra 2009), was also recommended. Guidance was given on hygienic measures to be taken during procedures likely to cause damage to the skin, such as castration, shearing and tattooing. Finally, any sheep already diagnosed with CLA had to be culled or managed separately for the duration of the trial.

Clinical examinations

Each sheep flock was visited at intervals of approximately six months during the trial period of three to four years. The regulations relating to the foot-and-mouth disease epizootic of 2001 meant that no farm visits could take place between February and November to the holdings that were in the trial during that year.

All trial sheep were individually identified through ear marks or tags and examined for clinical signs of CLA. This examination involved palpation and visual inspection of the superficial lymph nodes of the head, neck and body. Any sheep with enlarged or abscessed lymph nodes were isolated, and, where possible, samples of purulent discharge were taken for bacterial culture. At each visit, every sheep over six months of age was clinically examined in this way, after which it was blood sampled.

Blood samples were taken from sheep on farm A twice during 1999. The trial was then discontinued with the sale of the flock. Blood samples were taken from sheep on farm B on seven occasions between May 1999 and June 2003, on farm C on eight occasions between July 1999 and May 2003, on farm D on five occasions between July 1999 and August 2003, on farm E on five occasions between February 2000 and July 2003, and on farm F on five occasions between May 2000 and August 2003.

Serology

Sera were examined blind at the Dutch Central Veterinary Institute (ID-DLO), Lelystad, the Netherlands, using the refined ELISA method described by Dercksen and others (2000). On the basis of the ELISA results, samples were designated as negative, inconclusive or positive. When the ELISA test reaction was inconclusive, a western blot technique was applied to the sera. The western blot result was taken as positive when at least two bands were clearly visible on the test membrane at 31 kDa and 68 kDa. If the western blot gave a negative result (no bands) or an unconvincing result (one or two very weak bands), the sample was classified as inconclusive. All sheep classified as seronegative or inconclusive were retained within the flock and re-sampled at the next flock test. It was recommended that all sheep considered to be seropositive by ELISA or Western blot should be culled immediately or removed from the flock and managed separately.

TABLE 2: Site of caseous lymphadenitis (CLA) lesions in 28 sheep examined postmortem

Lesion site	Number (%) of sheep with CLA lesions
Lungs	13 (46)
Parotid lymph node	10 (36)
Bronchomediastinal lymph node	8 (29)
Prescapular lymph node	3 (11)
Submandibular lymph node	2 (7)
Liver	2 (7)
Prefemoral lymph node	1 (4)
Summary of the distribution of CLA lesions	
Internal CLA lesions only (lungs and/or visceral lymph nodes)	14 (50)
CLA lesions in superficial lymph nodes only	8 (29)
Both internal and superficial CLA lesions present	6 (11)

Pathology

During the trial, any sheep identified as CLA-positive on the basis of clinical examination or serology were either isolated from the rest of the trial flock or culled. A proportion of the sheep that were clinically affected and ELISA-positive were examined postmortem. Where flock management allowed, a proportion of the ELISA-negative cull sheep were also examined postmortem. A standard operating procedure was adopted to ensure consistency. Postmortem examinations were carried out by the authors. Sheep from flocks A, B, C and D were examined at the Scottish Agricultural College Veterinary Services Disease Surveillance Centre, St Boswells. Sheep from flocks E and F were examined at the Agri-Food and Biosciences Institute, Veterinary Sciences Division, Omagh. Each sheep was condition scored, and a general external examination of the carcase was performed, with particular reference to any enlarged superficial lymph nodes or purulent sinus discharges. A detailed postmortem examination was then undertaken, once again with particular reference to the lymph nodes and to the lungs.

Any suspect lesions, all prescapular, submandibular, parotid and retropharyngeal lymph nodes and a portion of bronchomediastinal lymph nodes were cultured and examined as described by Cowan and Steel (1993). Tissues that showed no gross lesions of CLA were dipped in 40 per cent alcohol and flamed to remove any surface contaminating organisms. They were stomached in nutrient broth, before plating out on to blood agar. Plates were incubated aerobically at 37°C and inspected at 24 and 48 hours. If *C pseudotuberculosis* was present, visible growth tended to be limited at 24 hours, with colonies reaching a diameter of 1 to 2 mm at 48 hours. Presumptive identification of recovered bacteria was made on the basis of colony morphology, catalase activity and microscopic examination after Gram staining. A definitive identification of the organism was made using the API Coryne identification system (bioMérieux).

Results

Clinical examinations

Before the first round of clinical examinations, 11 sheep in flock A had clinical signs consistent with CLA and were removed from the flock, and four sheep in flock B, one sheep in flock C, three sheep in flock D, five sheep in flock E and seven sheep in flock F were confirmed as clinically and bacteriologically positive for CLA. At the first round of testing, a further three sheep in flock B, 30 sheep in flock C and one sheep in flock D had clinical signs consistent with CLA. No further clinical cases of CLA were detected in flocks A, B, C, E or F during subsequent rounds of testing. However, the owner of flock D continued to report visible evidence of disease throughout this period.

Contact with the owner of flock B confirmed that the flock remained clear of clinical evidence of CLA until it was sold in 2005, and contact with the owners of flocks C, D, E and F as recently as July 2009 confirmed that those flocks also remained free of clinical disease.

Serology

The results of the serological examinations are summarised in Table 1. In all four flocks where testing was completed and management

advice on the control of infection was followed (flocks B, C, E and F), the serological incidence of infection declined. In flocks B and C, seroprevalence reduced to such an extent that at the final two flock tests no seropositive sheep were detected. At the final test, two sheep were seropositive in flocks E and F. These four animals were examined after culling, and no CLA lesions were found. The decline in seroprevalence in flock C during the trial is shown in Fig 1. In comparison, the owners of flock D failed to follow advice on the removal of seropositive sheep, and as a result seroprevalence and incidence of clinical signs increased during the trial. The seroprevalence in flock D is shown in Fig 2.

Pathology

A total of 88 postmortem examinations were performed on sheep of known serological status from the six trial flocks. Of these, 28 carcasses were found to have gross evidence of abscessation, subsequently confirmed as CLA by bacteriology. CLA lesions were most commonly seen in the lungs (13 cases), parotid lymph nodes (10 cases), bronchomediastinal lymph nodes (eight cases) and prescapular lymph nodes (three cases) (Table 2). Of the 13 sheep with lung abscesses, seven also had lesions in at least one other superficial or visceral lymph node site or within the parenchyma of the liver.

Discussion

Since the arrival of *C pseudotuberculosis* in the UK, the prevalence of CLA in the sheep industry has risen inexorably. The proprietary vaccines used widely to control CLA in other parts of the world have not been licensed for use in many European countries, including the UK. Farmers concerned about the spread of infection between and within flocks have therefore had to rely on clinical examination to identify affected sheep.

The development of an ELISA method for detecting antibodies to the PLD exotoxin of *C pseudotuberculosis* (ter Laak and others 1992) led to the establishment of a CLA health scheme within the Dutch dairy goat sector (Dercksen and others 1996). This scheme established a means for owners of infected goat herds to eradicate CLA, with the ultimate aim of CLA-free accreditation for their herd.

Although serological testing has been used as a research tool in a number of other countries, blood tests have not been widely used to control and eradicate disease. The present study has shown that the regular use of an ELISA to test and remove positive sheep, in association with clinical inspections and changes in farm management, can greatly reduce the incidence of CLA in affected flocks.

The proportion of sheep with gross lesions that showed only the internal or visceral form of CLA was somewhat higher (50 per cent) than in previous reports from the UK, which report that between one-quarter and one-third of sheep were affected in this way (Malone and others 2006, Baird and Fontaine 2007). Almost half of the visceral lesions identified in the present study were located within the lung parenchyma. Sheep with such pulmonary lesions have been identified by several authors as the principal source of infection for other sheep (Ellis and others 1987, Pepin and others 1994). The first mathematical model of *C pseudotuberculosis* infection indicated that, whereas transmission from superficial lesions predominates during the early spread of infection within a naive population, transmission from respiratory abscesses dominates the endemic phase of disease (O'Reilly and others 2008). This indicates that CLA management based purely on the removal of visibly affected sheep is unlikely to be successful. Thus, using serology to identify infected sheep offers the opportunity for enhanced disease control.

The most significant effects of the strategy were seen in trial flock C, where at the start of the study the seroprevalence was greater than 60 per cent and approximately 40 per cent of all adult sheep showed clinical signs of disease. Although seven rounds of testing over three years were required to remove all seropositive sheep from the flock, the two final tests, which took place six months apart, failed to identify any antibody-positive sheep, and no further clinical cases of CLA were detected by the owner during the next five years. Although clinical inspection alone cannot detect animals with the internal form of the disease, the absence of external signs of CLA for this length of time, in the view of the authors, constitutes effective eradication of disease. In future, postmortem examinations of fallen stock or abat-

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toir examinations of cull sheep may be suitable methods of disease monitoring in flocks that have adopted CLA eradication by regular ELISA testing.

The present study indicates that effective control of CLA may be achievable in flocks with a high prevalence through the use of serology and management changes. However, it also makes clear that a reduction in disease prevalence is not possible when owners are insufficiently motivated to make the required changes in management or are unwilling to remove clinically or serologically positive sheep. This was indicated in trial flock D, where advice on the control of infection and culling was not followed and the clinical and serological incidence of CLA increased during the study.

This trial was fully funded; flock owners were not required to pay for any testing or veterinary visits, and they received payment for animals that were culled and submitted for postmortem examination. This would not be the case for a test-and-cull programme operated commercially. Thus, it seems likely that the owners of CLA-affected flocks of high value and small size would be most likely to consider such a scheme. The scheme may appear less attractive to the owners of larger flocks, where the costs of testing, culling and sourcing replacements would be correspondingly greater.

In recent years, work in the UK has led to the development of a commercially available CLA ELISA test for sheep. This assay has similar levels of sensitivity and specificity to those reported for the ELISA used in the present trial. The introduction of the UK-derived test has prompted interest in the use of serology to control the spread of CLA within the UK sheep industry. In 2005, the CLA Monitoring Scheme incorporated the UK-developed serodiagnostic ELISA and western blot analysis based on the recombinant PLD antigen, to encourage presale testing of sheep (Anon 2005).

CLA eradication by means of ELISA testing may be hindered by the development of a vaccine against CLA. Although no fully licensed vaccine is available, vaccination against CLA is allowed in the UK with the authorisation of the Veterinary Medicines Directorate (VMD). With the agreement of the VMD, an autogenous *C. pseudotuberculosis* vaccine may be prepared. Alternatively, on receipt of a special treatment certificate, a proprietary CLA vaccine may be imported into the UK. Furthermore, anecdotal evidence suggests that unauthorised importation and use of proprietary vaccines may be occurring. This is significant because the use of CLA vaccines containing PLD antigen has been shown to produce seroconversion detectable as positive titres in the ELISA test (Fontaine and others 2006). For this reason, the use of vaccination is incompatible with a test-and-cull programme as described here.

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Vaccination confers significant protection of sheep against infection with a virulent United Kingdom strain of *Corynebacterium pseudotuberculosis*

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Abstract

Using a virulent United Kingdom *Corynebacterium pseudotuberculosis* isolate, an ovine experimental model of caseous lymphadenitis was developed, in which the manifestation of disease was equivalent to the naturally observed infection in this country. Subsequently, the capacity of several experimental vaccines to protect against experimental challenge was determined. Sheep were immunised with a recombinant derivative of phospholipase D, deriving from the virulent UK isolate, a formalin-killed bacterin of the same strain, or a bacterin supplemented with recombinant phospholipase D. Following homologous experimental challenge, the phospholipase D and bacterin vaccines were observed to confer statistically significant protection against infection, and appeared to restrict dissemination of challenge bacteria beyond the inoculation site in the majority of animals. More importantly, the combined vaccine succeeded in providing absolute protection against infection, whereby challenge bacteria were eradicated from all vaccinates. In addition to the experimental vaccines, a commercially available CLA vaccine, unlicensed for use in the European Union, was assessed for its capacity to protect against heterologous challenge. The vaccine conferred significant protection, although the dissemination of infection beyond the inoculation site was not restricted as it had been with the previous vaccines. However, no animals immunised with this vaccine manifested infection within the lungs; thus, a potentially important route of disease transmission was eliminated. The results of this study provide information pertinent to the development of an effective caseous lymphadenitis vaccination strategy in the UK.

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Keywords: *Corynebacterium pseudotuberculosis*; Caseous lymphadenitis (CLA); Phospholipase D (PLD); Bacterin; Vaccination; ELISA; Glanvac

1. Introduction

The facultatively intracellular bacterium, *Corynebacterium pseudotuberculosis*, is the causative agent of the chronic, suppurative disease of sheep and goats known as caseous lymphadenitis (CLA). The disease, which causes significant economic losses to the sheep industries worldwide [1–4], is characterised by the formation of encap-

sulated abscesses containing thick, caseous pus, most frequently within superficial lymph nodes, but also within internal lymph nodes and organs. Until recently, CLA was apparently absent from the United Kingdom; the first documented case of the disease in this country was reported in 1990 [5], following the importation of infected Boer goats in 1987 [6]. Subsequently, following the first sheep outbreak in 1991, CLA has become increasingly prevalent within the terminal sire breeds, where at least as many as 18% of flocks are thought to be infected [7]. In addition, through the sale of infected rams, the disease is threat-

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ening to become widely established within commercial flocks.

The potential to vaccinate sheep against infection with *C. pseudotuberculosis* has been the focus of research attention for many years now. Bacterin vaccines, or those comprised of cellular components have been shown to offer a degree of protection against experimental infection [8–12]. In addition, they have also shown some success when used to vaccinate against naturally acquired infection in sheep and goats [13,14].

Significantly, some of the earliest investigations into vaccination against *C. pseudotuberculosis* infection reported the capacity of the potent *C. pseudotuberculosis* exotoxin to induce protective immunity in guinea pigs [15]; however, despite these encouraging results, the general consensus was initially held that the chronic nature of *C. pseudotuberculosis* infections in sheep, compared to the relatively acute manifestation in guinea pigs, rendered exotoxin vaccines unsuitable for use against CLA [16,17]. Later work revealed that *C. pseudotuberculosis* exotoxin, now characterised as phospholipase D (PLD) [16], did in fact offer protection against infection in sheep [18]; subsequently, significant efforts have been made to produce effective CLA vaccines based on this antigen. The majority of studies have employed formalin-inactivated toxoid vaccines derived from PLD-rich *C. pseudotuberculosis* culture supernatants, and these have conferred varying levels of protective immunity in both sheep and goats [18–22].

At this time, there is no commercially available CLA vaccine licensed for use in the UK. Permission may be sought by veterinary practitioners for the use of so-called autogenous vaccines, which are adjuvanted bacterins derived from *C. pseudotuberculosis* isolates from flock outbreaks, intended only for immunisation of animals on the holdings from which the vaccine isolates derived; however, the protective capacity of these relatively crude preparations has been purely anecdotal. In light of these facts, there currently exists no information pertaining to the capacity to vaccinate against CLA in the UK. Interestingly, the clinical manifestation of the disease in this country has been suggested to be more similar to that of CLA in goats than that of sheep elsewhere in the world [23]; however, whether this is due to differences between *C. pseudotuberculosis* isolates, the susceptibility of UK sheep breeds or other differences in the pathogenesis of CLA infections in this country, is open to debate. We have assessed the capacity of a *C. pseudotuberculosis* bacterin to protect sheep against homologous challenge with a virulent

UK isolate of *C. pseudotuberculosis*. In addition, we have assessed the capacity of a recombinant derivative of PLD and a recombinant PLD-supplemented *C. pseudotuberculosis* bacterin to protect against equivalent challenge. Finally, we have also investigated the capacity for a commercial vaccine preparation, not licensed for use in this country, to protect against heterologous challenge with a UK *C. pseudotuberculosis* strain.

2. Materials and methods

2.1. Media and culture conditions

C. pseudotuberculosis was propagated on blood agar (BA) plates derived from Blood Agar Base (Oxoid) and 5% (v/v) sheep blood, or in Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, Hampshire, UK), at 37 °C (200× rpm for liquid cultures) for up to 48 h. Unless otherwise specified, *Escherichia coli* strains were routinely cultured at 37 °C (200× rpm for liquid cultures) in Luria Bertani medium (LB; Oxoid) containing ampicillin (100 µg/ml), or carbenicillin (50 µg/ml) and 10 mM glucose for expression experiments.

2.2. Molecular biological techniques

Unless otherwise specified, all DNA manipulations were performed according to standard techniques [24]. Restriction endonucleases and other modifying enzymes were purchased from Promega (Southampton, UK) or Roche (East Sussex, UK) and used according to the manufacturers' supplied instructions. Small-scale extraction and purification of plasmid DNA from *E. coli* was performed using the QIAquick Spin Miniprep Kit (QIAGEN, Crawley, West Sussex, UK), and genomic DNA was extracted from *C. pseudotuberculosis* using the NucleoSpin Tissue Kit (BD Biosciences (Clontech), Erembodegen, Belgium). PCR-amplified DNA fragments were purified using the Strataprep PCR Purification Kit (Stratagene, La Jolla, CA, USA), and cloned into pPCR-SCRIPT using the PCR-SCRIPT Cloning Kit (Stratagene). For cloning, restriction endonuclease-digested DNA fragments were electrophoresed through 1.0% (w/v) agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). Custom PCR and sequencing primers (Table 1) were synthesised to order by Sigma-Genosys (Sigma-Genosys Ltd., Haverhill, Suffolk, UK). Automated DNA sequencing was performed using a MegaBACE sequencing apparatus (Amer-

Table 1
Oligonucleotide primers

Primer name	Sequence ^a	Description
<i>pld</i> #01b	5'-CGCGCC <u>CATATGGCGCCTGTTGTGCATA</u> ACCCAGC-3'	Binds at bp 73–95 of <i>pld</i>
<i>pld</i> #02	5'-GCGCGGATCCTACCACGGGTTATCCGC-3'	Binds at bp 924–907 of <i>pld</i>
pET(fwd)	5'-GGATAACAATTCCCCTC-3'	Binds at bp 5261–5277 of pET-15b
pET(rev)	5'-CTAGTTATTGCTCAGCG-3'	Binds at bp 5452–5436 of pET-15b

^a Restriction endonuclease cleavage sites underlined; *pld*#01b = *Nde*I and *pld*#02 = *Bam*HI.

sham Biosciences UK Ltd., Buckinghamshire, UK) by the Functional Genomics Unit of the Moredun Research Institute.

2.3. Cloning of the *C. pseudotuberculosis* *pld* gene

The *pld* gene of the virulent UK *C. pseudotuberculosis* isolate, 3/99-5 [25] was amplified by PCR. The published *pld* sequence (GenBank accession #L16586) was used to design the primers *pld*#01b and *pld*#02, and to facilitate cloning primers included restriction endonuclease cleavage sites. The amplified fragment, corresponding to the truncated *pld* gene lacking the first 72 bp (encoding the 24 amino acid (aa) secretion signal sequence), was cloned into pPCR-SCRIPT and the resulting construct was designated pMMF003. To allow expression of the truncated 3/99-5 *pld* gene, pMMF003 was digested with *Nde*I and *Bam*HI, and the 859 bp fragment was ligated into pET-15b. Following transformation of *E. coli* XL10-Gold Kan (Stratagene), the desired construct was identified and designated pMMF004. DNA sequencing of both leading and complementary strands was performed using the primers pET(fwd) and pET(rev), to confirm the identity of the cloned fragment.

2.4. Expression and purification of recombinant PLD (rPLD)

Expression of the *pld* gene in pMMF004 was performed in *E. coli* BL21 (DE3) and induced by the addition of IPTG (1 mM final concentration) to exponentially-growing cultures (OD_{600nm} of ≈0.6). Induced cultures were incubated for 1 h prior to the addition of rifampicin (150 µg/ml final concentration). Incubation was continued for a further 3 h, and then cells were harvested by centrifugation at 6000 × *g* for 15 min at 4 °C. Cell pellets were resuspended in BugBuster Protein Extraction Reagent (Merck Biosciences (Novagen), Nottingham, UK) to approximately 5 ml/g of wet cell pellet. Complete protease inhibitor cocktail (Roche) was added (1 × final concentration) to inhibit proteolytic degradation, then Benzonase enzyme (Novagen) was added (1 µg/ml final concentration) and incubation was performed with gentle rotation on a shaking platform for 40 min at room temperature. During this incubation, cells were lysed and viscosity due to nucleic acids was reduced. Subsequently, centrifugation was performed at 16,000 × *g* for 30 min at 4 °C to pellet cell debris, and then cleared supernatants were supplemented with imidazole to a final concentration of 10 mM.

rPLD was purified by immobilised metal-ion affinity chromatography. Twelve ml EconoPak columns (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) were loaded with 2 ml (bed volume) of Ni-CAM HC resin (Sigma–Aldrich Company Ltd., Irvine, UK), pre-equilibrated with lysis buffer (Column Buffer (CB) [50 mM Tris–HCl, pH 8.0 and 300 mM NaCl] supplemented with 10 mM imidazole). To each column was added 5 ml of cell lysate (originating from approximately 500 ml of culture), and the volume was supplemented with a

further 5 ml of lysis buffer. Columns were sealed and incubated for 16 h at 4 °C on a tube rotator. Subsequently, columns were drained by gravity flow and washed with 25 × 2 ml of wash buffer (CB supplemented with 20 mM imidazole). Finally, column-bound protein was eluted in 5 × 2 ml of elution buffer (CB supplemented with 250 mM imidazole). Eluted proteins were concentrated and buffer exchanged in 10 mM PBS (pH 7.2), supplemented with 0.1% SDS and 1 × complete protease inhibitors, through 10K *M_r* Amicon Ultra centrifugal filters (Millipore, Watford, UK) at 3080 × *g* and 4 °C. The final concentration of rPLD was determined using the DC Protein Assay (Bio-Rad) and the purity of the preparation was estimated by analysis of SDS- and silver-stained polyacrylamide gels to be in excess of 99%.

2.5. Preparation of challenge inoculum

C. pseudotuberculosis 3/99-5 was passaged three times on BA plates, and then growth was collected using sterile, cotton-tipped swabs. Bacteria were suspended in PBS, in polystyrene Universal tubes, by gently rolling swab tips against the tube sides. Dilutions of the primary suspension were prepared to give the required cells/ml for subsequent challenge. Cells/ml were estimated by use of a Densimat (bioMérieux, Basingstoke, UK), and then quantified accurately using a Thoma counting chamber.

2.6. Preparation of formalin-killed *C. pseudotuberculosis*

For vaccination, *C. pseudotuberculosis* was cultured on BA plates, as for Section 2.5. Approximately 10¹¹ cfu were suspended in 20 ml of PBS in a Universal tube and supplemented with 40% formalin to a final concentration of 0.2% (v/v). Cells were incubated overnight at 4 °C, and then quantified using a Thoma counting chamber. Subsequently, cells were pelleted by centrifugation at 3080 × *g* for 15 min at 4 °C, and resuspended in distilled water to a final concentration of 1.25 × 10¹⁰ cells/ml. To confirm complete killing 100 µl aliquots of the bacterial suspension were plated onto BA plates, incubated at 37 °C and monitored daily for 5 days.

2.7. Experimental challenge and vaccination

For all challenge/vaccination experiments, male, approximately 1-year-old Suffolk-cross sheep were used. Prior to undertaking the vaccination study, a dose titration experiment was performed to determine the minimum cells/ml required to induce experimental infection manifesting clinical signs and pathology similar to naturally observed CLA. Groups of two animals were inoculated with 10², 10³, 10⁴ or 10⁸ bacteria, and an unchallenged control group was also included, administered sterile saline. The identification of the animals in each challenge group was 1578N + 1638N, 1630N + 1570N, 1549N + 1531N, 1689N + 1728N, and 1640N + 1603N, respectively. For chal-

lenge, a 1 ml inoculum was administered subcutaneously, 2 cm caudal to the base of the left ear of each animal, on a line passing through the ear to the left eye. Subsequently, animals were housed in their respective groups for 9 weeks, during which time blood was collected at regular intervals for serology. Animals were then sacrificed, a full necropsy was performed, and samples corresponding to inoculation site abscesses, lung, liver, spleen and brain tissues, as well as left- and right-hand-side (LHS and RHS, respectively) parotid, prescapular, sub-mandibular, retropharyngeal, mediastinal, prefemoral, inguinal and popliteal lymph nodes were collected for determination of the presence of bacteria. Subsequently, vaccinated animals were challenged with the determined optimal bacterial dose, exactly as described above, following which they were housed in their respective groups for 3 months until *post mortem* analysis.

For immunisations, vaccine antigens were formulated in physiological saline to final volumes of 1 ml, containing 50% (v/v) of a 13 mg/ml colloidal suspension of aluminium hydroxide (Alhydrogel; Sigma). Groups of six animals were immunised with either saline (control), 50% Alhydrogel (control), 50 µg of rPLD, 5×10^9 formalin-killed *C. pseudotuberculosis* whole-cells, or a combination of 50 µg of rPLD and 5×10^9 killed whole-cells; the Alhydrogel group was included to ensure that there was no effect of the adjuvant on the host resistance to infection. In addition, animals were immunised with the commercial inactivated-PLD vaccine, Glanvac-3™ (Commonwealth Serum Laboratories (CSL) Ltd., Victoria, Australia), currently unlicensed in the UK. Vaccines were administered subcutaneously to the RHS lateral neck. A 4-week interval was allowed between the first and second immunisations, and bacterial challenge was performed 2 weeks following the second immunisation.

2.8. *Post mortem* bacteriological analyses

To determine infection with *C. pseudotuberculosis*, tissue samples were disrupted in PBS; aliquots were plated onto BA plates and incubated to allow colony development. Presumptive identification of recovered bacteria as *C. pseudotuberculosis* was made on the basis of colony morphology, catalase activity, Gram-stain and appearance by microscopical analysis. A random selection of samples were also analysed using the API Coryne test (bioMérieux).

2.9. Serology

Animals' immunological responses to vaccination and/or infection were determined by measurement of serum anti-*C. pseudotuberculosis* and anti-PLD IgG by ELISA. Ninety-six well microtitre plates were coated with either rPLD (rPLD-ELISA) or whole *C. pseudotuberculosis* cells (WC-ELISA) and blocked with $1 \times$ TBS buffer containing 0.05% (v/v) Tween 20 (TBST) and 3% (w/v) Top-Block (Sigma). Subsequently, plates were incubated in the presence of experimental serum samples, diluted (1/200) in TBST containing

1% Top-Block. Plates were washed with TBST, and bound anti-*C. pseudotuberculosis* and anti-rPLD IgG was detected by use of a horseradish peroxidase-conjugated mouse anti-sheep/goat IgG monoclonal antibody (Clone GT-34; Sigma) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma). Colorimetric reactions were stopped and intensified with 2 M H₂SO₄, and the absorbance of each sample at 450 nm was subsequently determined.

2.10. Computational and statistical analyses

Analysis of DNA sequences and design of oligonucleotide primers was performed using the Clone Manager Professional Suite (Scientific and Educational Software Ltd., Durham, NC, USA). Unless otherwise stated, statistical analyses were performed using Genstat (8th edition; VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Because of the lack of independence between measurements made on the same animal, a repeated measures model was fitted to the ELISA data. The correlation structure was modelled using a power model and the parameters of the statistical model were estimated using the REML directive in Genstat. Several analyses were carried out on the necropsy data to determine differences in the extent and pattern of infection in the various tissues. A one-way analysis of variance (ANOVA) was used to investigate the mean percentage of tissues infected with *C. pseudotuberculosis* in the different groups. An angular transformation of the percentages was used to stabilise the variances within the groups and mean differences between groups were assessed using a least significant difference (LSD) approach. Three different patterns of infection were defined: no tissues infected, infection at the inoculation site only, and infection at the inoculation site and at least one other tissue. Animals were classified as having one of these three pattern types and a Fisher exact test was implemented, using the R language for statistical computing (available from <http://www.R-project.org>), to determine whether there was an association between groups and pattern types. The nature of the association was illustrated using correspondence analysis.

3. Results

3.1. Optimisation of infection model

In order to determine the optimal dose of *C. pseudotuberculosis* required to induce a similar disease manifestation in experimentally infected animals to that observed in natural cases of CLA, a dose titration experiment was performed. Prior to infection, the absence of clinical symptoms or circulating anti-*C. pseudotuberculosis* whole-cell and anti-PLD IgG was confirmed by clinical examination and ELISA analysis of blood samples taken from experimental candidates using the rPLD- and WC-ELISAs (Fig. 1); thus, the absence of natural infection within the flock was ensured.

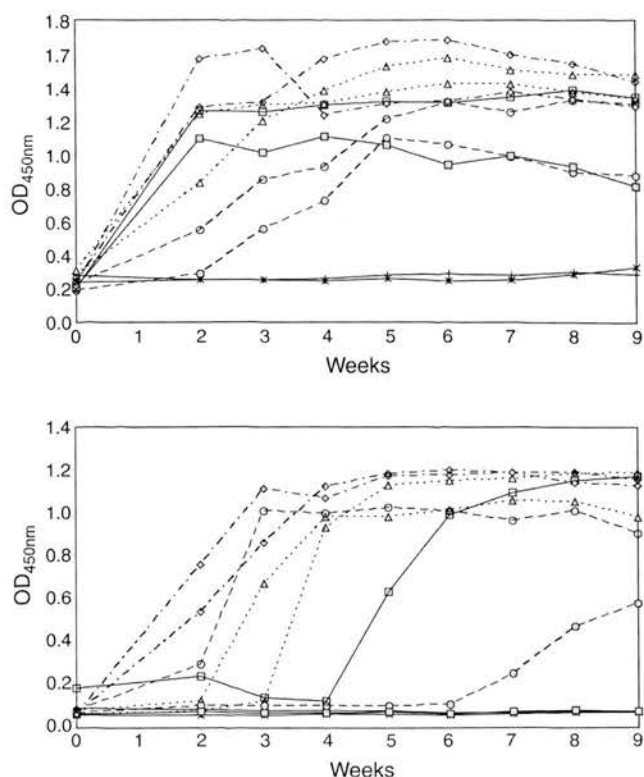


Fig. 1. Measurement of humoral immune response in sheep challenged with *C. pseudotuberculosis*. Levels of IgG (presented as OD_{450nm} values) against *C. pseudotuberculosis* whole-cells (top) and PLD (bottom) were determined. Challenge was performed at week 0, and groups correspond to: control, 1640N (*), 1603N (—+—); 10², 1638N (Δ) and 1579N (- - -Δ- - -); 10³, 1570N (○) and 1630N (-○- -); 10⁴, 1531N (—□—) and 1549N (□); 10⁸, 1728N (-◇- -) and 1689N (◇).

Bacteriological analysis of tissue samples taken *post mortem* was performed, and the sites from which *C. pseudotuberculosis* was recovered from each animal are presented in Table 2. In light of the observation that no bacteria were recovered from either brain, spleen or liver tissue samples or inguinal, popliteal and prefemoral lymph nodes from any of

Table 2
Recovery of *C. pseudotuberculosis* from tissues of challenged animals

Dose (cfu/ml)	Animal #	Tissue sample ^{a,b}					
		Lung	LPs.	LPa.	LR	Med	IS
Control	1640	-	-	-	-	-	-
Control	1603	-	-	-	-	-	-
10 ²	1579	+	-	-	-	-	+
10 ²	1638	+	-	-	-	-	+
10 ³	1630	+	+	+	+	-	+
10 ³	1570	+	-	-	+	-	+
10 ⁴	1549	+	+	-	-	+	+
10 ⁴	1531	-	+	+	-	+	+
10 ⁸	1689	+	+	-	+	+	+
10 ⁸	1728	+	+	-	+	+	+

^a LPs.: left-hand side (LHS) prescapular lymph node; LPa.: LHS parotid lymph node; LR: LHS retropharyngeal lymph node; Med: mediastinal lymph node; IS: inoculation site.

^b Presence/absence of bacteria denoted by +/-, respectively.

the experimental animals in the current study or in infection experiments conducted by us previously (unpublished data), future experiments were refined by eliminating the sampling of these sites. From the bacteriological data, it was apparent that as few as 10² cells were capable of causing infection. A challenge dose of 10⁴ cells was chosen for subsequent experiments, due to the observation that, with this dose, infection occurred in multiple lymph nodes and the lungs (*i.e.* was not restricted to a specific site).

Serological analysis of the blood samples taken from the experimental animals during the course of infection was performed. The levels of anti-*C. pseudotuberculosis* whole-cell and anti-PLD IgG of each animal were determined and are presented in Fig. 1 as the absorbance values at 450 nm, obtained using either ELISA. As expected, all infected animals were observed to have mounted an immunological response to the challenge bacterium, as determined using the WC-ELISA; however, there was no evidence that the magnitude of the response was directly associated with the number of challenge bacteria administered. Results were similar for anti-PLD IgG determination using the rPLD-ELISA; however, it is interesting to note that, despite mounting an immune response against *C. pseudotuberculosis* whole cells, one animal (1531N) failed to mount any observable response to PLD during the time-course of the experiment.

3.2. Vaccination and challenge of experimental animals with *C. pseudotuberculosis*

Prior to vaccination, experimental candidates were screened by clinical examination and by both ELISAs; all animals were free from observable signs of infection and lacked circulating anti-*C. pseudotuberculosis* whole-cell and anti-PLD IgG (data not shown). Unfortunately, one animal in group 3 died unexpectedly in the period between the first and second immunisations; however, no inoculation site reaction was observed, and the cause of death was determined not to be associated with any effects of vaccination.

Prior to experimental challenge, serology was performed to monitor the response to vaccination in each animal, using the WC-ELISA and rPLD-ELISA (Fig. 2, top and bottom panels, respectively). To compare immune responses between treatment groups, data are presented as average OD_{450nm} values (as determined by either ELISA) per group, and standard errors at each time point are included. As expected, using the rPLD-ELISA, the highest mean levels of anti-PLD IgG were observed for the animals immunised with rPLD (rPLD and rPLD + whole-cell vaccines) and GlanvacTM. For the former groups, anti-PLD IgG mean levels reached a maximum prior to the booster vaccination; furthermore, given the similar responses in animals in these groups, the whole-cell component of the rPLD + whole-cell vaccine was not considered to contribute to the immune response measured using the rPLD-ELISA. Mean levels of anti-PLD IgG in GlanvacTM vaccines were consistently less than those observed for rPLD and rPLD + whole-cell vaccines, and, rather than peaking, con-

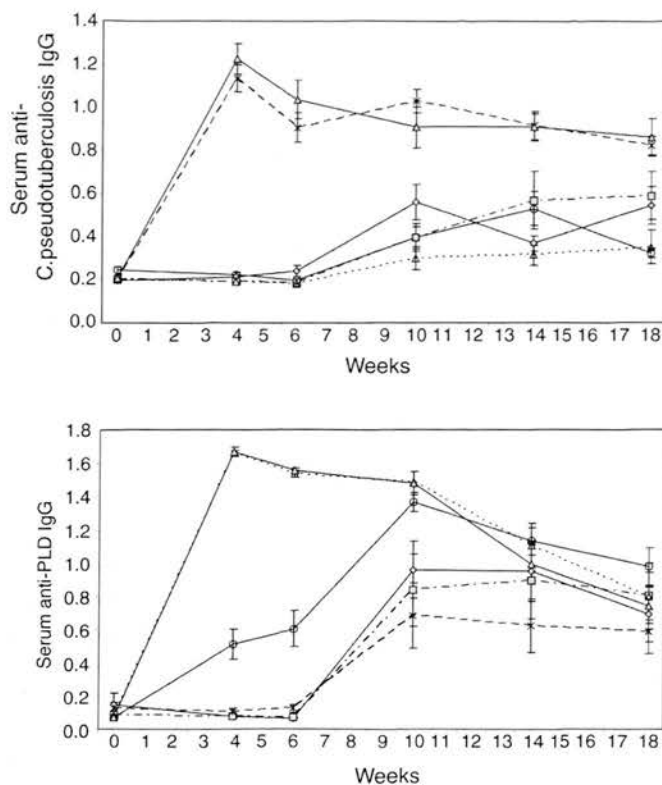


Fig. 2. Measurement of anti-PLD and anti-*C. pseudotuberculosis* IgG. Serum anti-*C. pseudotuberculosis* whole-cell (top) and anti-PLD (bottom) IgG levels were calculated for each treatment group, expressed as the mean and standard error of OD_{450 nm} per group. Samples were obtained following primary and secondary vaccinations (weeks 0 and 4, respectively) and for 3 months following bacterial challenge (at week 6). Treatment groups are (1) (---◇---) saline control; (2) (—□—) Alhydrogel control, (3) (Δ) rPLD, (4) (*) killed-whole cells, (5) (---Δ---) rPLD+killed-whole cells, and (6) (○) Glanvac-3.

tinued to rise following the secondary vaccination. Repeated measures models were fitted to both the whole cell and rPLD IgG ELISA data. For the rPLD data there was strong evidence ($P < 0.001$) of a different pattern in the mean responses over time, particularly where the levels of anti-PLD IgG in rPLD and rPLD+whole cell vaccinates were significantly higher than those of GlanvacTM vaccinates in the weeks immediately post-immunisation and pre-challenge. Mean levels of anti-PLD IgG in animals immunised with formalin-killed whole-cells were similar to those of non-immunised animals of the two control groups, confirming the observation that the immune response measured in rPLD+whole-cell vaccinates was likely to be a result of the rPLD portion of the vaccine (Fig. 2).

In contrast to results using the rPLD-ELISA, the WC-ELISA was able to determine the presence of high mean levels of anti-*C. pseudotuberculosis* IgG in killed whole-cell-vaccinated animals (whole-cell and rPLD+whole-cell vaccines), while not detecting an immune response in those animals vaccinated with rPLD or GlanvacTM (Fig. 2). This was probably due to the fact that PLD is a secretory antigen, which would have been sparingly present in the killed

whole-cell vaccine, and also in the cell preparations used in the WC-ELISA. Therefore, given the similarities in IgG levels between whole-cell and rPLD+whole-cell vaccinates, the response in animals immunised with rPLD+whole-cell vaccine can only have been against the whole-cell portion of the vaccine. As with rPLD and rPLD+whole-cell vaccinates (using the rPLD-ELISA), mean antibody levels in whole-cell and rPLD+whole-cell vaccinates reached their maximum level prior to administering the secondary vaccination. Overall, using the combined results of the two ELISAs, it was possible to determine that all animals, except for those of the control groups, had mounted observable immunological responses to vaccination.

The levels of serum anti-PLD and anti-*C. pseudotuberculosis* IgG were determined, as for the pre-challenge serum samples, following bacterial challenge at week 6 (Fig. 2). After challenge, average anti-PLD IgG levels in the two control groups increased to their maximum level by week 10 (4 weeks post-challenge). Anti-PLD IgG levels in killed whole-cell vaccinates remained similar to those of the control groups, rising steadily to a maximum level by week 10. Mean anti-PLD IgG levels in rPLD and rPLD+whole-cell vaccinates remained essentially constant until week 10, by which point anti-PLD IgG in animals immunised with GlanvacTM had reached an equivalent level; from that point on, mean anti-PLD levels in rPLD, rPLD+whole-cell and GlanvacTM vaccinates declined gradually to coincide with the anti-PLD levels observed in animals of the control and whole-cell immunised groups. Mean levels of anti-*C. pseudotuberculosis* IgG in the rPLD and rPLD+whole-cell vaccine groups remained essentially constant from the point of challenge. Mean anti-*C. pseudotuberculosis* IgG levels in the two control groups and animals immunised with rPLD and GlanvacTM were similar to each other, rising slightly after challenge, but never reaching an equivalent level to that observed for killed whole-cell vaccinates.

3.3. Post mortem analyses

Bacteriological sampling of tissue samples collected from vaccinated experimental animals was performed. The numbers of infected sites varied between groups, and to a lesser extent between individual animals. However, *C. pseudotuberculosis* was not recovered from the RHS parotid or the LHS and RHS submandibular lymph nodes of any animal. Data pertaining to the loci within animals of each treatment group from which bacteria were isolated is presented in Table 3. Significantly, no evidence of infection was found within any of the animals immunised with the rPLD+whole-cell vaccine, including the apparent resolution of inoculation site abscesses. The observed number of lung lesions varied to a large extent, both between groups and individual animals. Therefore, rather than directly comparing the actual numbers of lung lesions as a means of determining disease severity, a more robust method of comparing the presence or absence of lesions was used to determine differences.

Table 3
Recovery of *C. pseudotuberculosis* from tissues of vaccinated, challenged animals and percentage of infected tissues by treatment group

Treatment group	Group size (n)	Tissue sample ^a								Mean ^b	S.E.
		IS	LPa.	LPs.	RPs.	LR	RR	Lung	Med		
Saline	6	6/6	1/6	3/6	1/6	3/6	2/6	1/6	1/6	37.5	6.5
Alhydrogel	6	6/6	2/6	5/6	0/6	4/6	0/6	3/6	1/6	43.7	8.4
rPLD	5	5/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	15.0	2.5
Whole-cell	6	5/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	10.4	2.1
rPLD + whole-cell	6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0	0
Glanvac TM	6	5/6	1/6	1/6	0/6	3/6	0/6	0/6	0/6	20.8	7.0

^a Abbreviations correspond to: tissues; IS, inoculation site, and lymph nodes; Lpa., LHS parotid; LPs., LHS prescapular; RPs., RHS prescapular; LR, left retropharyngeal; RR, right retropharyngeal; Med, mediastinal.

^b Mean percentage of tissues containing CLA lesions.

An ANOVA was performed to determine whether the mean percentage of tissues infected with *C. pseudotuberculosis* varied significantly between the different treatment groups. Only the eight tissues observed to show infection in any of the animals were used in the analysis (RHS parotid excluded). There was strong evidence ($P < 0.001$) that the mean percentage of tissues infected was different between the different treatment groups. The mean percentages and standard errors on the original scale are shown in Table 3. The two control groups had similar means and there was no evidence of an affect of Alhydrogel on the outcome of infection. Furthermore, the means of the control groups were both significantly different to those of the groups of immunised animals (using an LSD at the 5% level), confirming that all of the vaccine antigens assessed in the study offered a significant level of protection against infection.

In the experimental subjects, the most common sites of infection were the LHS prescapular and retropharyngeal lymph nodes, which is consistent with the drainage of challenge bacteria from the site of administration to a local draining lymph node. To determine whether spread of *C. pseudotuberculosis* from the inoculation site to other internal sites was more or less likely within the different treatment groups, each animal was assigned to one of three different pattern groups (data not shown), which were determined on the basis of there being no tissues infected (P1), infection in the inoculation site but no other tissues (P2), or infection in the inoculation site and at least one other tissue (P3). A Fisher exact test indicated that there was very strong evidence ($P < 0.001$) that some treatment groups displayed different patterns of infection to other groups.

The association between treatment group and pattern type was investigated further using correspondence analysis (data not shown). The scores of the first and second components were plotted against each other for treatment groups and pattern types separately (Fig. 3). It was expected that if a pattern type (P1, P2 or P3) was associated with a particular treatment group then the pattern and treatment groups would be located close to each other on the correspondence plot. It was observed that P1 and rPLD + whole-cell vaccinates were clearly associated with each other and separated from the other groups on the first component. Furthermore, on the

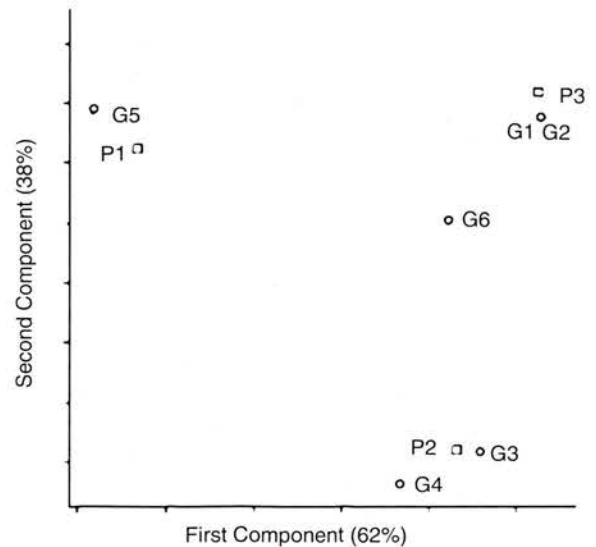


Fig. 3. A correspondence analysis plot showing treatment groups and pattern of infection. Treatment groups were saline (G1), Alhydrogel (G2), rPLD (G3), formalin-killed whole-cells (G4), rPLD + killed whole-cells (G5) and Glanvac-3 (G6). Patterns of infection were defined as no tissues infected (P1), infection in the inoculation site but no other tissues (P2) and infection in the inoculation site and at least one other tissue (P3). All treatment groups associate with a particular pattern of infection, with the exception of G6.

second component, P2 and P3 were distinct, with rPLD and whole-cell vaccinates associated with P2 and control animals associated with P3. Interestingly, GlanvacTM vaccinates did not cluster well with any pattern group, but associated most closely with P3.

4. Discussion

To our knowledge, this is the first study conducted to investigate the potential for vaccination of sheep in the UK against CLA. We report here statistically significant levels of protection against both homologous and heterologous challenge with a virulent, UK-derived *C. pseudotuberculosis* strain, isolated from a natural CLA outbreak. In a homologous challenge model, vaccination with either the highly purified rPLD antigen, or formalin-killed *C. pseudotuber-*

culosis cells prevented spread of challenge bacteria beyond the site of inoculation in the majority of immunised animals. More importantly, vaccination with a combination of rPLD and killed whole-cells resulted in complete protection against challenge. In addition, vaccination with the commercial vaccine, GlanvacTM, also offered statistically significant protection against heterologous challenge, although it was less effective in preventing the dissemination of challenge bacteria beyond the site of inoculation than the former antigens.

Experimental infections of sheep with *C. pseudotuberculosis*, conducted by us previously (unpublished data) and as part of this study, have revealed a consistent pattern of infection which appears to be restricted to the lymph nodes of the head and neck region and the lungs. This experimental data is consistent with the observed manifestation of CLA in the UK [23], which is uncommon in other countries. Interestingly, there is variation in the extent of lung involvement in our experimental candidates, which also appears to be consistent with the manifestation of CLA in the UK, in which up to 25% of infected animals may suffer from lung lesions [26]. The experimental infections undertaken in this study were conducted using the lowest number of bacteria as was possible to achieve signs of infection consistent with those observed naturally (10^4 cells, although 10^2 cells were found to cause infection); this has relevance, since excess numbers of bacteria may overwhelm the host immune response and mask any protective effects of vaccination.

PLD appears to be an important virulence factor, responsible for the dissemination of *C. pseudotuberculosis* from the primary site of infection [27]. Previous studies have assessed the capacity of different forms of PLD-based vaccine to protect against CLA [20,21,28,29], and in this study a highly purified, recombinant 6× histidine-tagged fusion derivative of PLD, lacking the secretion signal peptide was used. Despite being prepared under native conditions, the final rPLD vaccine formulation contained a small amount of detergent, which abrogated toxin activity (data not shown). Of the five rPLD-immunised animals remaining at the time of challenge, dissemination of the challenge bacteria beyond the site of inoculation was prevented, with the exception of one animal which also manifested evidence of lung infection.

Several authors have suggested that the protective immunity induced by vaccination with PLD derives from toxin neutralisation through the action of anti-PLD antibodies [19,21,30,31]. However, one study reported that a genetically inactivated PLD derivative did not confer a level of protection equivalent to that of GlanvacTM [29]; the latter vaccine comprising a formalin-inactivated PLD preparation derived from ultra-filtrated *C. pseudotuberculosis* culture supernatants [29,32]. The reason for the difference in protective ability was hypothesised to result from the remaining, approximately 1% [33], PLD activity following toxoiding, which potentially contributed to immunity via another route [29]. Given the high-purity and inactive nature of our rPLD vaccine, it is most likely that its protective capacity

was as a direct consequence of the stimulation of humoral immunity, since the alternative route of protection suggested above would be lacking in the absence of toxin activity, and PLD-based vaccines have not been shown to stimulate cell-mediated immunity [29]. Significantly, conflicting hypotheses as to the issue of the route of protection may well have their origins in the purity of vaccine preparations. Toxoid vaccines prepared from *C. pseudotuberculosis* culture supernatants routinely contain other, undefined bacterial antigens [32]; therefore, stimulation of protective immune responses by these other antigens may well contribute to the overall protection observed. In support of this, immunoblots using sera from GlanvacTM-vaccinated animals were observed to intermittently recognise a novel 40 kDa *C. pseudotuberculosis* serine protease [34], suggesting that this protein was present in some batches of vaccine. The antigen was shown to be located within *C. pseudotuberculosis* culture supernatants, and it is possible, therefore, to hypothesise that it may have been co-purified along with the toxin component during vaccine preparation.

Previously, it has been observed that the kinetics of the ovine antibody response to immunisation with PLD tend to be low until the point of bacterial challenge [35,36]. In the present study, the antibody response to vaccination with GlanvacTM corresponds to previous observations, in that mean anti-PLD IgG increased with successive vaccinations, and increased even further following challenge (Fig. 2). In contrast, animals vaccinated with rPLD mounted a statistically significantly more rapid response, which, on average, peaked prior to the second immunisation. The reason for the differences in the speed of the immune response between vaccines is not apparent, but inadvertent measurement of trace *E. coli* contaminating proteins in the rPLD preparation was shown not to contribute to the antibody response measured in the rPLD-ELISA by repeat testing of selected sera by ELISA using an HPLC-purified rPLD preparation (data not shown). It is possible that the different responses were due to different relative amounts of rPLD and wild-type PLD in the experimental and GlanvacTM vaccines, respectively. Alternatively, it has been reported that native PLD is more immunogenic than the toxoided form [32]. GlanvacTM comprises a fomol-toxoid while the rPLD antigen described here was inactivated by the presence of a small amount of detergent. Given that the two methods of inactivation differed, it is possible that rPLD remained more immunogenic than PLD in GlanvacTM.

Currently in the UK, the only vaccination strategy for which permission may be sought is for the use of so-called "autogenous" *C. pseudotuberculosis* bacterin vaccines. Autogenous vaccines are flock-specific, in that they are prepared using isolates from defined flock outbreaks, and are permitted for use only in the single holding from which they originated. Until now, evidence for the protective capacity of autogenous CLA vaccines in the UK has been purely anecdotal; however, we have now demonstrated significant protection against homologous challenge using a *C. pseudotuberculosis* bacterin vaccine. Our results are bolstered by previous stud-

ies, conducted in other countries, where bacterin vaccines have been successfully used to protect mice and sheep against experimental [8,9] and natural [14] infection by *C. pseudotuberculosis*. According to our results, vaccination with killed whole-cells was sufficient to prevent the spread of *C. pseudotuberculosis* beyond the site of inoculation, and in one animal even the inoculation locus was found to be sterile. While our data provides evidence that the use of autogenous vaccines may be efficacious against CLA, in our laboratory PFGE analysis of *C. pseudotuberculosis* isolates deriving from different animals of the same flock have revealed the presence of different, albeit related, clones in different animals (unpublished data). The genome of *C. pseudotuberculosis* of sheep/goat origin would appear to be extremely stable, and we have not observed alterations in genetic structure following multiple passages in synthetic media or through sheep ([25] and unpublished data). Therefore, the reason for the existence of multiple clones within a flock can only be as a result of the movement of animals between holdings, such as through normal sale/purchase practices. This has important implications for the success of autogenous vaccination strategies, should they be found not to confer protection against heterologous *C. pseudotuberculosis* strains.

The best CLA vaccine, whether it be formulated using *C. pseudotuberculosis* killed whole-cells, toxoided PLD, or a combination of both, has been the focus of debate for some years now. Previous studies revealed that the use of whole cell [11,37] and toxoid [18] vaccines, while conferring significant protection, were not capable of inducing complete protection against experimental infection with *C. pseudotuberculosis*. Latterly, Eggleton and co-workers published a report in which it was stated that the incomplete protection observed with these early experiments resulted from a lack of a suitable animal disease model at that time, and it was reported that the addition of whole-cells to a toxoid vaccine made no difference to the extent of the observed lesions following experimental infection [33]. However, in a contrasting study [32], it was claimed that a combined whole-cell/toxoid vaccine was capable of conferring complete protection against experimental challenge, and that this combination vaccine outperformed a toxoid vaccine equivalent to that used by Eggleton et al. [33].

The results of our study would tend to support the findings of previous authors [11,37], in that neither our rPLD or whole cell vaccine was sufficient to completely prevent CLA following experimental challenge. In contrast, in our hands, immunisation of sheep with a combination of rPLD and formalin-killed whole-cells resulted in complete protection against experimental challenge, inasmuch as no evidence of infection was identified in tissue samples from challenged animals. Even the infected sites of inoculation, which frequently remained as encapsulated, suppurative foci containing live *C. pseudotuberculosis* cells in other experimental groups, were able to resolve, and these observations are consistent with those of a previous study [32].

The reason for our combined vaccine offering such effective protection against challenge is possibly due to stimulation of a strong antibody-mediated response, combined with priming of the cell mediated immune system through subcutaneous vaccination with the killed whole-cells (although no attempt was made to measure the latter during any stage of the experiment). Interestingly, both the rPLD and the formalin-killed whole-cell vaccines prevented dissemination of challenge bacteria beyond the site of inoculation to essentially equivalent extents. The protection offered by rPLD was antibody-mediated; therefore, given that the magnitude of the humoral response to whole-cell antigens (following vaccination with either the killed whole-cell vaccine or the combination rPLD + whole-cell vaccine) was equivalent in scale to that observed against rPLD, it is also possible that complete protection derived purely as a result of humoral immunity deriving from vaccination with both antigens. In the absence of neutralising antibody, *C. pseudotuberculosis* has been shown to survive killing by non-activated macrophages of several species [38,39] and the importance of activated macrophages in the suppression of *C. pseudotuberculosis* growth and lesion development has been shown in a murine model [40,41]. In addition, murine macrophage killing of *C. pseudotuberculosis* following opsonisation with hyperimmune ovine serum has been demonstrated [38] and PLD has been shown to aid bacterial dissemination through promoting vascular permeability [27]. Taken together, these observations help explain the results of the combined rPLD/whole cell vaccination, since neutralisation of PLD (and other *C. pseudotuberculosis* proteins) by serum antibodies in vaccinated animals would presumably abrogate vascular permeabilisation to some extent (limiting the spread of the challenge bacterium) in addition to opsonising bacteria for killing by non-activated macrophages. Furthermore, if cellular immunity was involved in the protection observed in whole-cell vaccinates, adequate priming of the cell-mediated immune response would allow activated macrophages to kill bacteria already restricted to the site of inoculation by neutralisation of PLD (and other colonisation factors).

It seems likely that challenge bacteria were killed at the site of primary infection, soon after administration, since their dissemination would have resulted in the formation of granulomas within the draining lymph node and other sites. This hypothesis is consistent with that of Simmons and co-workers, who observed that sheep vaccinated subcutaneously with live, attenuated (AroQ-deficient) mutants of *C. pseudotuberculosis* remained unable to clear the vaccine strain from the lymph node draining the inoculation site [42]. It was considered likely that the inaccessible location of the attenuated mutants within the granulomas allowed their persistence through exclusion of immunological factors.

The data presented here is the first scientific investigation of the capacity for GlanvacTM to protect against the UK form of CLA. We have measured protection against disease

through its use, and, although the protection observed was lower than with the other experimental vaccines tested, it is of relevance that it was the only vaccine tested for its capacity to protect against heterologous challenge. Unfortunately, for this reason it is not possible to directly compare the protective capacity of Glanvac™ with our rPLD vaccine. It is interesting to note that, although the overall prevalence of infected loci was reduced within Glanvac™ immunised animals, there was only one animal which appeared free from infection. The remaining animals manifested infections of either the inoculation site, or several distinct loci. Therefore, there is the suggestion that this vaccine was not sufficient to prevent the dissemination of *C. pseudotuberculosis* beyond the primary site of infection, which is in contrast to observations of the rPLD and killed whole-cell vaccines. Interestingly, no lung lesions were identified in Glanvac™-vaccinated animals. An aerosol route of *C. pseudotuberculosis* transmission has previously been postulated [43], and it is therefore possible that, despite not completely preventing infection, the eradication of a potential route of disease transmission could have a significant impact on the spread of infection to other animals. This hypothesis is consistent with the findings of an Australian study, in which Glanvac™-vaccinated sheep with CLA were observed to have 96% fewer lung abscesses compared with unvaccinated infected sheep [44]; in that study, 77% of disease transmission occurred around the fourth and fifth shearings in the absence of any obvious discharging abscesses, implying spread from discharging lung abscesses. In light of these facts, it would appear that there is some justification for the use of the Glanvac™ range of vaccines against CLA in the UK; however, it is also entirely possible that a more effective alternative could be developed.

One question that remains to be answered is whether the difference in protection between Glanvac™ and our experimental vaccines was simply due to issues with the heterologous versus homologous challenge models, or due to the fact that Glanvac™ was simply outperformed by our experimental vaccines. This is relevant, since a single vaccine, if chosen wisely, might have the capacity to protect against infection by a variety of different isolates, irrespective of their geographical origin. Clearly, protection against heterologous challenge by our experimental vaccines needs to be determined, and future experiments are planned to address this. In conclusion, the data we have presented here contributes significantly to the existing knowledge regarding the potential for vaccination against CLA in the UK, and provides a firm platform from which to embark on future studies.

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