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 carcase 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 1	2	3	4	5	6	7	8	9	Total number of seropositive sheep
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.

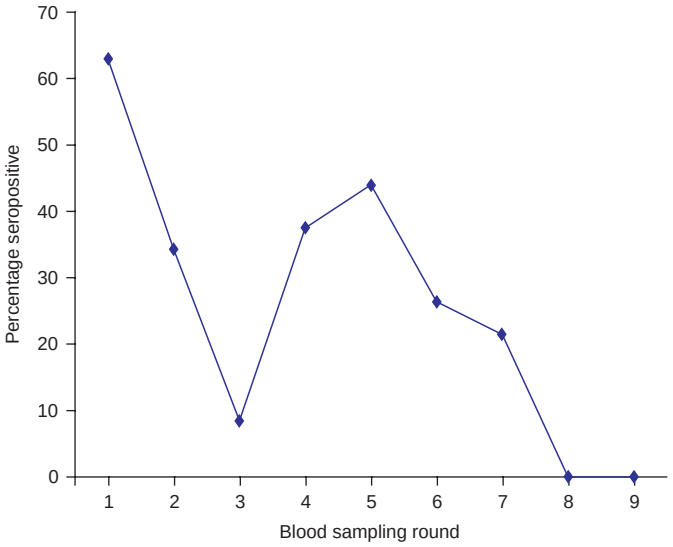


FIG 1: Seroprevalence of caseous lymphadenitis in flock C during the eradication trial

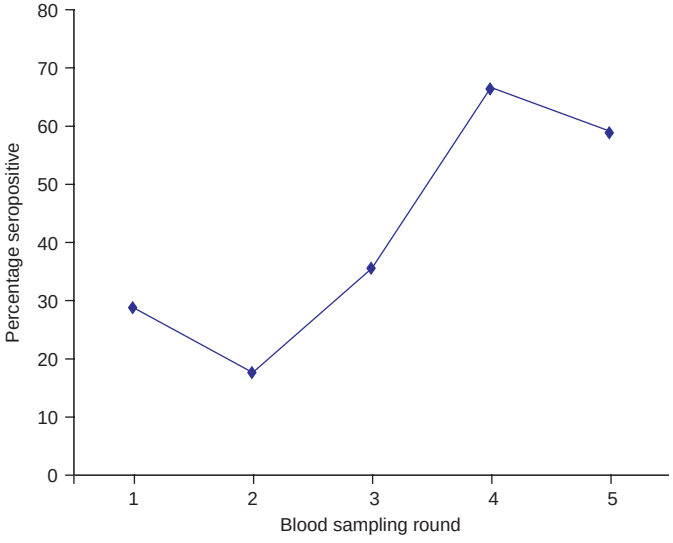


FIG 2: Seroprevalence of caseous lymphadenitis in flock D during the eradication trial

The source of the CLA infection was considered to be a shearling 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 shearling 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 crossbred ewes. The first cases of CLA were diagnosed in July 1999 and involved two shearling 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 carcases 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-

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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