BOVINE PARATUBERCULOSIS: INVESTIGATION OF AFFECTED CARCASSES AT MEAT INSPECTION

by

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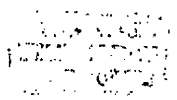
DEDICATION

To Mum.



SUMMARY

Crohn's disease is a chronic, progressive granulomatous enteritis of young humans. Analogies have been made with Johne's disease of cattle, especially following the isolation of the organism, *Mycobacterium avium subsp. paratuberculosis*, from some Crohn's patients. Much research has been conducted to define the aetiological agent of Crohn's disease. However, the cause remains unclear.

The aim of the project was to investigate the likelihood of clinical cases of Johne's disease passing meat hygiene inspection, following current fresh meat legislation and guidelines.

Carcasses considered fit for human consumption underwent post-mortem examination to investigate gross pathological changes. The extent of spread of the organism was investigated by cytological and histological examination and also by polymerase chain reaction of lymph nodes, intestines and selected tissues.

All the confirmed Johne's disease carcasses "set" and would pass meat hygiene inspection.

The most consistent findings at post-mortem were dilation of the mesenteric and gut lymphatics, and thickening of the terminal ileum, with "cobblestoning" of the ileal mucosa.

The most significant laboratory result was a prescapular lymph node of animal 136334 being positive for IS900, a genetic element of DNA unique to the organism. Moreover this animal had numerous granulomatous lesions throughout many lymph nodes and organs in the body, suggesting systemic spread of the organism.

The second part of this thesis describes a pilot study, conducted in an over thirty month scheme abattoir, investigating the application of a more thorough examination of the carcass within the abattoir. It was found that inspection of the gut mucosa was feasible within the time constraints of the slaughterline and if conducted within the gut room, posed minimal threat to hygiene.

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DECLARATION

The work in this thesis was carried out by the author, except where duly acknowledged, and has not been submitted for the award of a degree at any other university.

Hayley Haining

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INTRODUCTION

Johne's Disease.

Johne's disease, first described by Johne and Frothingham in (1895)[i], is a chronic non-caseating granulomatous enteritis of ruminants, deer (Riemann, Zaman, Ruppaner, Aalund, Jorgensen, Worsaae and Behymer 1979; Chiodini and Van Kruiningen 1983), rabbits (Greig, Stevenson, Perez, Pirie, Grant and Sharp 1997), foxes and stoats (Beard, Henderson, Daniels, Pirie, Buxton, Greig, Hutchings, McKendrick, Rhind, Stevenson and Sharp 1999). In cattle, Johne's disease is characterised by progressive emaciation, particularly of the gluteal muscles, and persistent homogenous diarrhoea; the affected animal usually has a good appetite until the terminal stages of the disease. The causative agent is *Mycobacterium avium* subsp. *paratuberculosis (M. a. paratuberculosis)*, a Gram positive, acid/alcohol-fast bacterium that posseses a robust waxy cell wall containing mycolic acids that renders it very resistant to the body's immune system (Gyles and Thoen 1986).

Johne's Disease in Cattle.

Johne's disease occurs worldwide and causes important economic losses (Riemann and Abbas 1983; Chiodini, Van Kruiningen and Merkal 1984a; Benedictus, Dijkhuizen and Stelwagen 1987). It is generally thought that cattle are infected via the faecal-oral route, although transplacental infection may occur more frequently than was first thought (Alexejeff-Goloff 1929, abstract in Journal of Comparative Pathology 1935; Pearson and McClelland 1955; Lawrence 1956; Doyle 1958; Kopecky, Larsen and Merkal 1967). Most recently, 24.6% of faecal culture positive pregnant cows had infected foetuses. This was the first time the incidence was studied (Seitz, Lawrence, Hueston, Bech-Nielsen, Rings and Spangler 1989). Experimental studies have shown that animals

infected early in life are most susceptible to developing the disease (Hagan 1938; Doyle 1953; Rankin 1958,1961 and 1962; Larsen, Merkal and Cutlip 1974) and so it is likely that dam to calf transmission occurs in most cases.

There are three potential scenarios following infection: (i) immunity or resistance, (ii) subclinical disease or (iii) clinical disease. The average age of cattle developing clinical signs is 2-5 years (Doyle and Spears 1951). The course of clinical disease is characterised by rapid weight loss and homogenous diarrhoea, which is resistant to therapy (see Figure 1, page 4). In nearly all cases the animal retains a bright demeanor and a good appetite until the terminal stages of the disease, at which time they may become pale, anaemic and develop submandibular oedema, and weakness (Radostitts, Blood and Gay 1994). Clinical signs are often exacerbated by calving and the onset of milking, or some other similar stressor (Chiodini et al. 1984a).

Pathologically, the disease is described as a chronic, granulomatous, non-caseating enteritis most commonly affecting the terminal portion of the ileum, ileocaecocolic junction and caecum, although any region of the intestine may be affected. There is little correlation between the clinical signs and the apparent severity of the pathological lesions (Jubb, Kennedy and Palmer 1993). Gross post-mortem examination generally reveals weight loss with loss of carcass fat characteristically preceding abdominal fat loss. Classically, the mesenteric lymph nodes are described as always being enlarged and pale with oedema. Lymphangitis is common and dilated lymphatics can be traced as thickened tortuous cords over the intestinal serosa and the mesentery. Dilated lymphatics are often the only visible pathology at gross post-mortem

Figure 1. Case 135524, a five year old cow showing faecal staining and weight loss with a provisional diagnosis of Johne's disease, subsequently confirmed at post-mortem.

examination and are considered reliable for a tentative diagnosis (Jubb et al.1993). There is diffuse thickening of the intestinal mucosa which is often thrown into transverse rugal folds that cannot be flattened out on stretching termed "cobblestoning" (see Figure 2, page 6). This is due to the infiltration of large numbers of inflammatory cells, predominantly lymphocytes, plasma cells and macrophages, into the submucosa and mucosa. The mucosal surfaces may be slightly reddened due to congestion and may have petechial haemorrhages. Well-developed lesions can be seen microscopically as granulomatous, non-caseating transmural enteritis. The villi can show varying degrees of atrophy; the crypts are distorted and some are dilated with cell debris or mucus filling their lumens. Epithelioid macrophages form swathes and whorls within the submucosa. Lymphangitis is one of the most consistent features and lymphatic vessels can be seen surrounded by lymphocytes and plasma cells. Lymph nodes show histiocytosis of the subcapsular sinus with variable infiltrates of epithelioid macrophages which, may replace much of the cortex and extend into the medullary sinuses (Jubb et al.1993). On tissue sections stained with Ziehl-Neelsen (ZN) stain, cherry-red, acid-fast bacilli may be seen arranged in a "chinese letter" configuration within the cytoplasm of macrophages. The infection may be disseminated (Alexejeff-Goloff 1929; Matthews 1930; Doyle 1954 and1958; Pearson and McClelland 1955; Lawrence 1956; Kopecky et al.1967; Larsen, Stalheim, Hughes, Appell, Richards and Himes 1981; Hines, Buergelt, Wilson and Bliss 1987; Sweeney, Whitlock and Rosenberger 1992) and organisms have been demonstrated in the mononuclear cell compartment of blood, suggesting spread via the lymphatic system and venous drainage (Koenig, Hoffsis, Shulaw, Bech-Nielsen, Rings and St-Jean 1993). Although the acid-fast bacilli are not always visible on ZN-stained sections, the organism is regularly identified

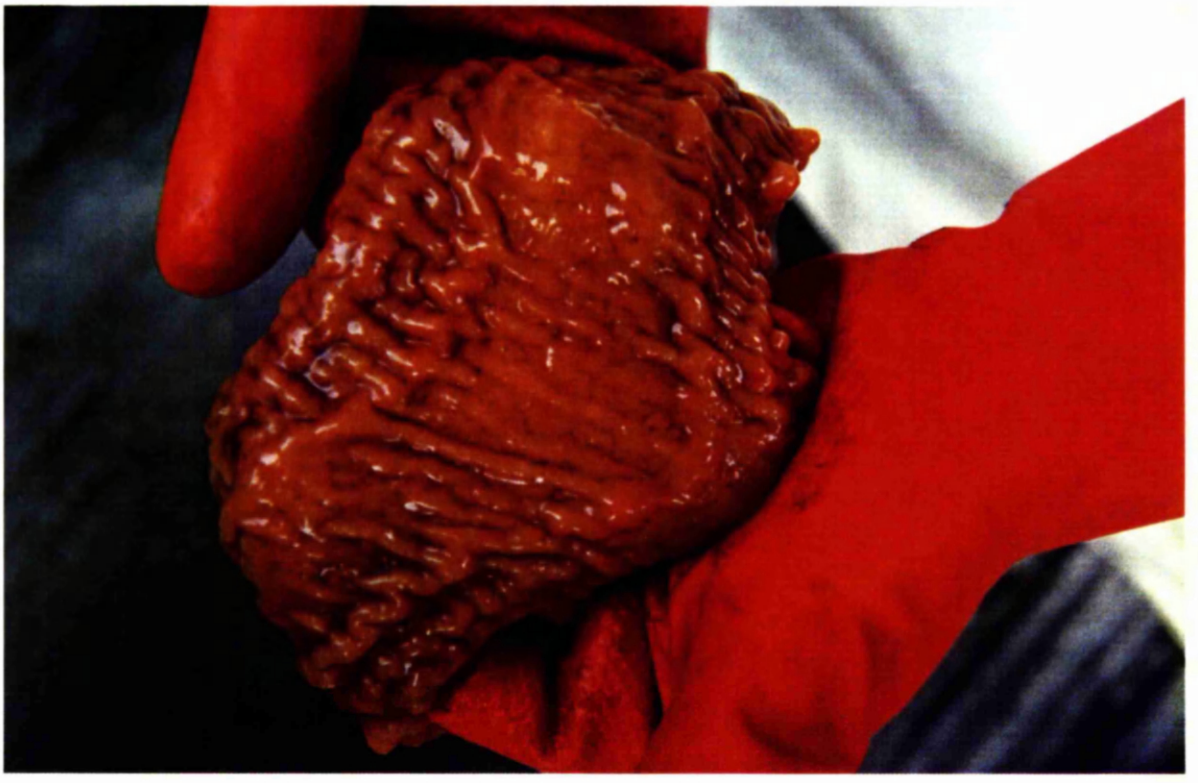

Figure 2. An archive slide demonstrating "cobblestoning" of the terminal ileum represented by prominent transverse rugae which could not be flattened out when stretched (kindly provided by Dr. H. Thompson, University of Glasgow).

from these acid-fast negative samples using other techniques. Polymerase chain reaction (PCR), immunohistochemical and electron microscopy techniques have been used in sheep (Clarke and Little 1996; Perez, Garcia Martin and Badiola 1996), and by electron microscopy in cattle (Condron, Shroen, Black, Ridge and Hope 1994). The presence of organisms can be demonstrated in many organs in disseminated disease using an immunoperoxidase technique (Nguyen and Buergelt 1983). Reports of remote lesions (i.e. not directly related to the intestinal tract) with acid-fast organisms in sheep and goats that were either experimentally or naturally infected have been reported, most commonly in the liver and lungs and less consistently in the hepatic lymph nodes, kidneys and spleen (Harding 1957; Rajya and Singh 1961; Nakamatsu and Fujimoto 1968).

The prolonged and irregular incubation period of Johne's disease, the ability of some animals to remain subclinically infected, even though they continue to shed the organism in faeces (Rankin 1954; Merkal, Larsen, Kopecky and Ness 1968), and the difficulty in confirming the subclinical disease in life (Chiodini et al. 1984a) has made the study of the incidence of the disease problematic. In the 1950s, when the Central Veterinary Laboratories carried out a study of wastage and disease in cattle, the incidence of Johne's disease in Surrey, Berkshire and Wiltshire in the period 1950 -1956 was 0.84% (methods of diagnosis not reported)(Withers 1959). Also, a series of eleven studies throughout England and Scotland during 1953/54-1956/57, revealed the incidence of the diseases was 0.42% (Withers 1959). The incidence of latent infection from samples taken from apparently normal cattle slaughtered at abattoirs ranged from 6-17% (Taylor 1949,1952; Rankin 1954; Smith 1954, summarised by Withers 1959).

More recently, a practice-based study in the south-west of England estimated that the proportion of affected farms in this region was 1% with the cumulative incidence (the proportion of non diseased individuals at the beginning of a period of study that become diseased during the period of study) of infected herds 1.9% per annum (Cetinkaya, Egan and Morgan 1994). A postal study of England and the Welsh borders reported that 17.4% of farms had experienced at least one animal with the disease sometime previously and 4.9% of farms having at least one case during the period of 1985 to 1994. At the same time, 6.3% of farms in the southern regions of England reported having a case during this period (methods of diagnosis not reported) (Cetinkaya, Erdogan and Morgan 1998).

Another report from the same workers described an abattoir based study of adult cattle and some young stock (age not specified). Intestinal lymph nodes were sampled with 3.5% being positive using a PCR technique and 2.6% positive for *M. a. paratuberculosis* by culture on Herrold's egg yolk medium (Cetinkaya, Egan, Harbour and Morgan 1996). This was lower than reported by Withers in 1959 and it has been suggested that lower figures may be due to improved nutrition (Julian 1975; Larsen and Merkal 1968), lower incidence of Channel Islands breeds in the national herd or sampling technique (Cetinkaya, Erdogan and Morgan 1997). The practices of removing calves from infected mothers and immediate removal and culling of suspect animals may also have contributed to the reduced incidence (Julian 1975; Merkal 1984).

The Diagnosis of Johne's Disease.

The diagnosis of Johne's disease in life presents several difficulties, but contrary to popular belief, many accurate diagnostic methods exist.

Problems arise due to the fastidious growth requirements of *M. a. paratuberculosis*, the paradoxical immune response towards the infection with an initial cell mediated immune response, followed by a humoral response (before clinical signs develop), and lastly anergy when the immune response becomes exhausted.

Several serological tests exist. The agar gel immunodiffusion test (AGID) is most often used for rapid diagnosis of clinical cases (Sherman, Markham and Bates 1984; Sweeney and Whitlock 1990). The test relies on the antibody production towards the organism and so, the use of this test is restricted to animals in the later stages of the disease when the antibody response begins (Ridge, Morgan, Sockett, Collins, Condron, Skilbeck and Webber 1991). An antibody response is not detectable during the first year of the disease (Parker 1988).

More frequently used is the enzyme-linked immunosorbent assay (ELISA), especially in conjunction with another diagnostic test. The specificity of the ELISA was greatly increased by preadsorption of test sera with *M.phlei* which reduced the occurrence of false positives by removing any cross reacting antibodies to other mycobacteria and other environmental organisms which share common antigens (Yokomizo, Yugi and Merkal 1985). A range of sensitivity values have been reported at between 15% and 57% for subclinically affected animals and 88% for clinical cases shedding high numbers of the organism in faeces (Sweeney, Whitlock and Buckley 1995). Improved sensitivity has been found to correlate with increased shedding of the organism in faeces (Yokomizo, Kishima, Mori and Nishimori 1991).

The complement fixation (CF) test is used less frequently as it has lower specificity than both the AGID and ELISA tests (Stabel 1998). False positives can occur due to cross reaction with *Actinomyces spp.*, *Dermatophilus*, *Nocardia*, and *Streptomyces*

(Chiodini et al. 1984a). False negatives may occur due to the development of tolerance (cell mediated immune responses are susceptible to tolerance development (Davis, Dulbecco, Eisen and Ginsberg 1980)), anergy and antigen masking factors (Bendixen 1978).

It has been suggested that the intradermal Johnin and the intravenous Johnin tests should not be used for diagnosis, control or prepurchase testing as the organism shares many antigens in common with environmental mycobacteria, eg; *M. avium*, compared to *M. bovis* and *M. tuberculosis* (Collins 1996).

The first and strongest response to mycobacterial infection is by T lymphocytes (Collins 1996). In vitro assays can be used to measure the production of interferon γ (Wood, Corner and Plackett 1990). The advantage of this test is the ability to detect subclinically affected animals in a herd.

As an alternative to serological tests, detection of the organism may be attempted but this also presents problems. Examination of ZN-stained faecal smears has limitations as animals may only shed organisms intermittently and other saprophytic bacteria may stain acid-fast, eg; *Nocardia*, *Rhodococcus*, and other *Mycobacteria* (Holt, Kreig, Sneath, Staley and Williams 1994). For this reason acid-fast bacteria must be seen in clumps for a diagnosis of Johne's disease (Chiodini et al. 1984a).

Culture and confirmation of Mycobactin dependency, has traditionally been the definitive method of diagnosis but *M. silvaticum* and some primary isolates of *M. avium* are also Mycobactin dependent (World Organisation for Animal Health 1996). *M. a. paratuberculosis* can be considered an obligate pathogen of mammalian cells and so culture and definitive identification from clinical samples is 100% specific (Collins 1996). This

technique is limited by the fastidious growth requirements of *M. a. paratuberculosis*; the organism is best grown on enriched egg yolk agar containing Mycobactin J (Francis, Madinaveitia, Macturk, and Snow1949; Snow 1954; Merkal and McCullough 1982). Growth in culture generally takes 12-16 weeks but contamination by moulds and bacteria are common and the decontamination process may injure the bacteria and so retard growth or even inhibit growth altogether (Merkal and Thurston 1968). Furthermore, the method of decontamination of samples can significantly affect the sensitivity of this method (Chiodini et al. 1984; Collins 1996). Although a highly specific method, culturing is not wholly reliable as approximately 50% of infected animals may be undetected after one test (Parker 1988; Sanftleben 1990; Sweeney and Whitlock 1990; Sockett, Carr and Collins 1992). Culture has the disadvantage of expense of labour and materials, and also expertise may vary greatly between laboratories.

At post-mortem, histopathology using a ZN stain on sections is also highly specific for the presence of *M. a. paratuberculosis*. However, bacilli are not always detected in lesions and lesions may not be uniformly distributed throughout the intestine (Jubb et al. 1993).

PCR targeting of IS900, (a genetic element of DNA) is being increasingly used for further identification of the organism from both solid and liquid media (Cousins, Evans and Francis 1995; Whittington, Marsh, Turner, McAllister, Choy, Eamans, Marshall and Ottoway 1998). The PCR allows the many fold amplification of DNA in vitro. The sequence of the desired gene must be known so that two oligonucleotide "primers", complementary to the sequence can be used. Oligonucleotide "primers" are added to the PCR reaction mix, which includes the DNA target. The mixture is heated to denature the DNA and as the mixture cools an excess of "primers" ensures most target strands will anneal to the

primers. The "primers" flank either side of the target. The DNA polymerase of *Thermus aquaticus* extends the "primers" using the target as a template. After 20-30 cycles of denaturing, annealing and extension, the yield of DNA can be increased 10^6 to 10^9 fold (Madigan, Martinko and Parker 2000).

The target DNA used with *M. a. paratuberculosis* is a genetic element, repeated about 18 times within the genome of the organism. This stable repeated genetic element is an insertion element and has been labelled IS900 (Collins, Gabric and DeLisle 1989; Green, Tizard, Moss, Thompson, Winterborne, McFadden and Hermon-Taylor 1989). Several workers have designed "primers"(Vary, Anderson, Green, Hermon-Taylor and Mcfadden 1990; Moss, Sanderson, Tizard, Hermon-Taylor, El-zaatari, Markesich and Graham 1992). Culture of the organism with evidence of Mycobactin dependency and identification of IS900 is the most definitive method of diagnosis (Thoen and Haagsma 1996).

Mycobacteria

Mycobacteria are slim rod shaped, non-motile, non-sporeforming, weakly Gram positive organisms that are strictly aerobic. Their cell wall contains N-glycolmuramic acid and has a high lipid content of up to 60%. This gives them a hydrophobic surface, causes them to stain poorly with standard dyes and may be part of the reason for their slow growth rate in culture as nutrients have difficulty diffusing through the cell wall (Sharma and Adlakha 1996). The addition of Tween 80 to cultures "wets" the surface and increases the speed of growth (Sharma and Adlakha 1996). The bacteria stain by heating with carbol fuschin and once stained can only be decolourised by acidified alcohol. This property is used to distinguish Mycobacteria from other organisms and ZN is the stain commonly used.

M. a. paratuberculosis is a short rod 0.5μm wide and 1-2μm in length, although it may be shorter when grown *in vitro*. The bacteria are generally found in clumps on smears from infected animals, although single organisms can be found. The organism grows very slowly on egg yolk agar with glycerin extracts of *M. phlei* or *M. a. paratuberculosis* (laboratory-adapted) and the optimum temperature for growth is 38-39°C. Primary cultures take at least 16 weeks to grow, although subculturing may improve growth. The organism may persist in pastures for about one year and is susceptible to sunlight, drying and high soil pH (Carter and Cole 1990).

As an alternative to solid medium, BACTEC Middlebrook medium (Becton Dickinson) is a liquid broth containing egg yolk medium, with vancomycin, amphotericin B, nalidixic acid and Mycobactin J. It is a radiometric growth system which has been shown to detect growth of mycobacteria earlier than conventional culture systems. The indicator of growth is the release of $^{14}CO_2$ from metabolised radiolabelled substrate (palmitic acid). This method has the advantage that growth of mycobacteria can generally be detected within seven weeks of inoculation and the lowest amount detected is ten mycobacteria/gram of tissue (Sockett et al. 1992).

A further well-described aspect of culturing mycobacteria is the development of spheroplasts. Spheroplasts are atypical morphologic forms derived from classical bacteria and are a phenomenon seen in many different species of bacteria (Feingold 1969). They have a defective or absent cell wall. The bacterial cell wall is important for cell division, regulating flow of molecules into and out of the cell and plays a part in determining the pathogenicity of the organism. The universal feature of the bacterial cell wall is a peptidoglycan or muramylpeptide polymer, which imparts a fair degree of rigidity and

support to the cell (Feingold 1969). Compounds of the cell wall are thought to be heavily involved in determining the virulence of the cell by having the ability to resist cellular and humoral defences and also by being toxic to some host tissues (Feingold 1969). The mycobacterial cell wall contains 6,6 trehalose dimycolate that is thought to be leukotoxic, and sulfatides which are thought to promote the survival of the bacilli in macrophages (Gyles and Thoen 1986). The cell wall of some bacteria is also responsible for the production of endotoxin from the lipid moiety of the lipopolysaccharide in the Gram negative bacteria and from the peptidoglycan moiety of Gram positive bacteria. Many whole bacteria secrete small molecules, which are chemotactic for polymorphonuclear leukocytes, endotoxin and complement and so are powerful stimuli for the inflammatory response (Feingold 1969).

It could be considered that spheroplasts would, at first glance, with their incomplete cell wall and loss of antiphagocytic properties and susceptibility to low osmolality, be very fragile and unlikely to survive. However, they lack surface antigens, which stimulate the inflammatory response and can therefore remain in the body in protected environments (Feingold 1969). Whether spheroplasts are pathogenic remains a subject of much study. There is evidence that the spheroplasts of some organisms continue to synthesise and secrete compounds into their environment, eg. the L-form (spheroplast) of *Clostridium tetani* continues to secrete exotoxin (Schiebel and Assandri 1959). Furthermore, an experimental study of enzootic pneumonia in pigs found pneumonic lesions of piglets after inoculation of L-forms of *Haemophilus parainfluenzae*. In 7/9 piglets, only L-forms were obtained at post mortem, but the pathological lesions present were identical to those caused by parent forms. The major

difference was that disease caused by L-forms was more protracted (McKay, Abelseth and Vandreumel 1966). Other authors have reported that granulomatous disease occurred in mice injected with only L-forms of *M. tuberculosis* and in this instance granulomatous endocarditis and myocarditis was produced without any evidence of reversion to classical forms (Merline, Golden and Mattman 1971).

In contrast, a study inoculating L-form tubercle bacilli into guinea pigs resulted in isolation or culture of *M. tuberculosis* from all animals showing disease. Those from which only L-forms were retrieved had no lesions nor were positive on tuberculin test (Ratnam and Chandrsekhar 1976). From these studies it has been proposed that only parent forms of bacteria are pathogenic (Ratnam and Chandrsekhar 1976), although Ratnam noted that in his own study, the disease in guinea pigs was more virulent than would be expected for the dose given. He proposed that one L-form might give rise to multiple parent bacteria. This phenomenon would explain acute recrudesence of disease if there was accelerated reversion to the parent form as is seen in acute progressive leprosy (Chatterjee 1965).

Interestingly, spheroplasts have been implicated in chronic, recurrent renal disease in humans. Even after therapy, spheroplasts have been found in the renal medulla, where conditions are naturally hypertonic, and then secreted in the urine (Clasener 1972).

However, despite being implicated in various cases, the exact pathogenicity of spheroplasts is unknown. It is possible that bacterial spheroplasts may remain in the body without producing clinical disease. Alternatively, their low chemotactic activity and slow growth could maintain a subclinical or chronic disease. Thus it is possible that the extended period of time taken to culture *M. a. paratuberculosis* from a case of Johne's disease may be due to the time taken for reversion of spheroplasts to the parent form of

the bacteria. The existence of spheroplasts would also explain the lack of acid-fast bacilli in stained sections in some apparently affected animals. Cell wall deficient organisms have been detected by electron microscopy in subclinically infected cattle, with paucibacillary enteritis (Condron et al.1994) and ovine clinical cases of paratuberculosis with no demonstrable acid-fast organisms which have then been identified as *M. a. paratuberculosis* by immunological techniques (Stamp and Watt 1954; Clarke and Little 1996; Perez et al.1996).

Crohn's Disease.

Crohn's disease is an idiopathic, chronic, ulceroconstrictive, inflammatory, transmural bowel disorder of man in which the affected bowel feels thickened and neighbouring mesenteric fat and serosa may be affected by the inflammatory process (Price 1992).

The first recognisable description of Crohn's disease was from a report by Dalziel (1913) who described a condition observed in several patients which was a form of chronic enteritis very similar in some ways to that of intestinal tuberculosis but also different in a number of respects. Dalziel was the first to compare the disease to Johne's disease, which had recently been reported. The observations of Dalziel were contested by those of Ignard, who believed that these atypical lesions should be considered tuberculous (Ignard 1913 cited in Mosckowitz and Wilensky 1923). The disease was definitively described by Crohn, Ginzberg and Oppenheimer (1932) as a chronic, low grade inflammation of the terminal ileum with no visible acid-fast bacilli on histopathology.

Patients with Crohn's disease suffer from chronic weight loss, abdominal pain, diarrhoea and constipation, vomiting and general malaise. They generally present with symptoms in their teens to early twenties (Kirsner 1991). Seventy to eighty percent of patients require surgical treatment (Goligher, deBombal and Burton 1972).

Macroscopically there are three basic patterns of lesions:

(i) ulceration - serpiginous clefts, discontinuous or in their earliest form as aphthous lesions on the mucosal surface.

(ii) strictures - short, long, multiple or single. A hosepipe stricture at the terminal ileum is the classic appearance.

(iii) "cobblestoning" where the thickened mucosa is divided by fissures between raised sections of mucosa, infiltrated by the inflammatory process.

Histopathologically a variety of characteristics may be seen. The most reliable diagnostic feature is the presence of epithelioid cell granulomas with the variable presence of giant cells. These are present in 50-70% of all cases but the numbers of granulomas vary greatly, rectal lesions having the highest numbers and decreasing in number in more proximal intestinal sites. Serpiginous clefts extend from the mucosal surface to the submucosa and often as far as the serosa. Even in the absence of the above characteristics, Crohn's disease has a distinctive transmural inflammatory pattern that can be distinguished from other forms of inflammatory bowel disease on histopathology. The submucosa is often widened by oedema or is fibrotic. Focal aggregates of lymphocytes are often present as a bead-like line on the serosal surface. The goblet cell population is relatively normal and crypt alignment remains intact (Price 1992). Granulomatous

Crohn's lesions have also been found within skin, muscle and lung (Kirsner and Shorter 1983). Metastatic Crohn's disease can be found within the skin and liver (Price 1992).

Spheroplastic phase mycobacteria have been isolated from patients with Crohn's disease (Chiodini, Van Kruiningen, Thayer and Coutu 1986). A significant finding from patients with Crohn's disease is the isolation of atypical cellular material that did not stain with conventional acid-fast dyes. Restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA genes revealed an identical genetic pattern to that of *Mycobacteria* sp. (Chiodini et al. 1986). This material was found to be spheroplasts. Further spheroplasts were isolated from 16 of 26 Crohn's patients but not from 13 patients with ulcerative colitis (UC) or 13 patients with other diseases of the bowel (Chiodini et al. 1986). Again, the relationship between the cell wall deficient form and the parent form of the organism was proved by RFLP (Chiodini et al. 1986).

The aetiology of Crohn's Disease.

The aetiology of Crohn's disease still remains unclear. Infectious agents, genetics and dietary causes have all been proposed. A genetic cause has been suggested after two studies in different countries demonstrated that first degree relatives are at a greater risk than the general population (Mayberry 1980; Monsen 1990). Furthermore, another study reported that 4/6 monozygotic twins had concordant Crohn's disease, but in none of the dizygotic twins was concordant Crohn's disease found (Monsen 1990). Studies from Tel Aviv and Beer Sheva have shown the incidence within the Jewish population to be seven fold higher than in the local non-Jewish population; indeed, Crohn's disease in the local Bedouin Arab population is considered very rare (Odes, Fraser and Hollander 1989, Odes, Fraser, Krugliak, Feynes, Fraser and Sperber

1991). Significantly, however, studies of the Jewish population as a whole suggest the aetiology involves both polygenic inheritance and environmental factors (Mayberry, Judd, Smart, Rhodes, Calcradt and Morris 1986, Sonnenberg 1986). Most recently, a preliminary report of a study in Florida has found evidence for the presence of *M. a. paratuberculosis* in the breast milk of two women with Crohn's disease (Naser, Schwartz and Shafran 2000, cited by Hermon-Taylor 2000). If confirmed this could provide supportive evidence to account for the familial tendency of Crohn's disease (Hermon-Taylor 2000).

An infectious aetiology for Crohn's disease is still pursued. Numerous studies have attempted to identify viruses and L-form bacteria (Chiodini 1989). The first serious attempt to isolate mycobacteria from Crohn's tissue was that of Van Patter (Ph.D. thesis 1952). Acid-fast organisms were isolated from 3 of 43 patients (7%) from a total of 1762 samples. Seven different media were used and some were cultured for 15 months. The positive cultures took 6, 7.5 and 8 months to grow but the organisms could not be subcultured or identified (cited in Chiodini 1989).

In 1978, mycobacteria were proposed as the putative agent of Crohn's disease when *M. kansasii* was isolated from the intestinal lymph node of a patient with Crohn's disease and material suggestive of cell wall deficient organisms (spheroplasts) was isolated from 22/27 Crohn's patients , 7/13 with UC and from 1/11 controls with non-inflammatory bowel disease (non-IBD) (Burnham and Lennard-Jones 1978). However, the role of *M. kansasii* as the causative agent was questioned as it is a well known opportunist pathogen causing disease in people who are already debilitated by chronic disease (Wallace, Swenson, Silcox, Good, Tschen and Stone 1983). Subsequent studies using indirect fluorescent antibody techniques found that, 9/11 Crohn's patients, 8/10 UC

patients and 0/20 control patients were positive for *M. kansasii* (White, Nassau, Burnham, Stanford and Lennard-Jones 1978), further implicating Mycobacteria in the pathogenesis of IBDs and Crohn's disease.

In 1984, interest in the mycobacterial aetiology of Crohn's disease heightened following the report that two strains of *M. a. paratuberculosis*-like organisms had been isolated from 11 Crohn's patients but not from 3 patients with UC or 3 patients with non-IBD (Chiodini, Van Kruiningen, Thayer, Merkal, and Couto 1984b). These workers also reported the isolation of spheroplasts, in culture, from 16/26 patients with Crohn's disease, 4 of which then transformed into the parent organism *M. a. paratuberculosis*. On culture spheroplasts were not isolated from 13 patients with UC or 13 patients with non-IBD. Further analysis of this material by RFLP successfully identified these atypical cellular forms as *M. a. paratuberculosis* (McFadden, Butcher, Chiodini and Hermon-Taylor1987). Previously DNA:DNA hybridisation was used as a means of identification but this procedure was unable to distinguish between some members of the *M. avium-intracellulare* complex because of >90% homology of their DNA (McFadden et al.1987).

Chiodini et al. reported some animal susceptibility studies in 1984. The isolates they obtained from human Crohn's patients were used for experimental infections. Mice inoculated intravenously with viable bacilli in saline, developed non-caseating granulomas of the liver, spleen and mesenteric lymph nodes. Mice inoculated intraperitoneally with identical inoculum developed hepatic granulomas with acid-fast bacilli present in the granulomas (Chiodini et al. 1984b).

Similar studies inoculating young chickens produced granulomas in 3 different groups. In the first group inoculated by the oral route, all 6 birds developed intestinal

granulomas; 3 of 5 birds in the group inoculated intracardially developed granulomas in different organs (the pericardium, gall bladder and liver in two; the third had multiple granulomas in the lungs) and in the last group, inoculated intraperitoneally, four of the six birds developed granulomatous peritonitis; two had granulomas of the lung and two had granulomas of the liver; one had a solitary granuloma of a caecal tonsil (Van Kruiningen, Ruiz and Gumprecht 1991).

In addition oral inoculation of a newborn goat with viable cultured bacilli (strain Linda) produced granulomatous enteritis without acid-fast bacilli after 5months (Van Kruiningen, Chiodini, Thayer, Coutu, Merkal and Runnels 1986). There was only a very short lived IgM response to suggest seroconversion and although the response to *M.a. paratuberculosis* purified protein derivative (P.P.D.) was greater than that to the P.P.D. of *M. tuberculosis,* the level of reactivity would not normally be considered positive (Van Kruiningen et al. 1986).

The recovery of *M. a. paratuberculosis* from 86% of Crohn's subjects in a recent study using liquid culture (7H9 broth) and PCR has indicated further advances in the diagnostic methods and adds more weight to the evidence that Crohn's disease does have a mycobacterial aetiology (Schwartz, Shafran, Romero, Piromalli, Biggerstaff, Naser, Chamberlin and Naser 2000).

Possible transmission of *M. a. paratuberculosis* to man.

Previous studies have shown that *M. a. paratuberculosis* can be cultured from the milk samples of clinically affected and subclinically affected cattle (Taylor, Wilks and McQueen 1981; Sweeney et al. 1992) and that a major source of infection to young calves is the milk of their infected mothers (Chiodini1989). One report claimed that 7%

of retail milk samples were positive for IS900 in the south and southwest of England. Of these 81% were positive in the cream and pellet fractions, which was interpreted to be indicative of intact organisms rather than free DNA. Fifty percent of the IS900 positive samples and 16% of the negative samples were positive on culture after up to 40 months incubation (Millar, Ford, Sanderson, Withey, Tizard, Doran and Herman-Taylor 1996). It was, therefore, proposed that a very slow growing residual population of *M. a. paratuberculosis* could be present in milk after pasteurisation. The resultant hypothesis was that "repeated exposure may result in the cumulative acquisition of a resident population and, after many months or years, may lead to paucimicrobial enteritis" (Millar et al. 1996).

An identical study (Bickley, Grant, Rajagopalan, O'Riordan, Pope and Parkes 1998) found that *M. a. paratuberculosis* cells were found in all three centrifugal fractions of spiked milk samples. The lack of a signal or weaker signals in some fractions obtained by Millar et al. (1996), was thought to be due to the different amounts of inhibition to PCR occuring in the pellet, cream and whey fractions. This study found that IS900 could be detected in these fractions and that magnesium and calcium ion levels were critical for the success of the PCR (Bickley et al. 1998).

Further work has subsequently investigated the efficacy of pasteurisaton methods in eliminating viable *M. a. paratuberculosis* organisms from milk. Pasteurisation was instituted for commercial milk in the late 1900s to destroy milk borne pathogens, in particular *Mycobacterium bovis. M. a. paratuberculosis* has been found to be more heat resistant than *M.bovis* (Grant, Ball, Niell and Rowe 1996). Using spiked milk samples, the high temperature short time (HTST) method of pasteurisation was found to cause a

10^5 to 10^6 fold reduction in numbers of *M. a. paratuberculosis* at a spiking dose of 10^7 cfu/ml (colony forming units/millilitre). A 10^2 to 10^3 fold reduction was obtained when the sample was spiked with10^4 cfu/ml. Acid-fast survivors were found on both the holder method (63.5°C for 30 minutes) and the HTST method (71.1°C for 15 seconds). However the results of these tests using an experimental fascimile of commercial methods should be interpreted with care as a greater thermal inactivation is thought to be achieved if samples are agitated during the pasteurisation process as occurs in commercial practice (Grant et al. 1996). Also, the inoculate level of 10^4 cfu/ml can be considered to be the levels expected in milk in a worst case scenario and the level 10^7 cfu/ml is therefore clearly excessive (Grant et al. 1996). Grant et al. (1996) suggested that pasteurisation under laboratory conditions is not suitable to completely eliminate *M. a. paratuberculosis* organisms from milk when it is present in large numbers. A second study by the same author found that *M. a. paratuberculosis* to survive HTST pasteurisation when at levels of 10^2-10^3 cfu/ml in milk prior to heat treatment (Grant, Ball and Rowe 1998). The authors suggested the BACTEC media used to culture the cells was more conducive to isolation of injured organisms. Also static HTST pasteurisation techniques mean that milk particles (fat globules and somatic and bacterial cells) may form, the cores of which cannot be guaranteed to reach the required temperature (Grant et al. 1998).

Studies on the inactivation of organisms of the *Mycobacterium avium-intracellulare complex* (to which *M. a. paratuberculosis* is closely related) in meat have also shown that these organisms were much more heat resistant than *M.bovis*. For any given treatment period *M. bovis* was destroyed at temperatures 6-7°C lower than those for organisms of the *M. avium- intracellulare* complex (Merkal and Whipple 1980). Studies

on the heat inactivation of *M. avium-intracellulare* organisms within "hotdogs" (Merkal and Whipple 1980) revealed that the three most important parameters during cooking were

(i) peak internal temperature (at least 75^0C)

(ii) time in the killing range (over 60^0C for at least 5 minutes)

(iii) relative humidity.

Those studies used meat spiked with *M. avium* serovar 10c, a very heat resistant strain. It was found that when using radiant heat or oil cooking, temperatures of 75^o C (peak internal temperature) with at least 5 minutes above 60^0C were needed to cause a 10^5 decrease in number of viable organisms. As a result the author expressed concern over the practice of precooking sausages where rapid heating is used to reduce moisture followed by rapid cooling and freezing as this allows the product to be in the killing range for only a very short period of time (Merkal, Crawford and Whipple 1979).

From these studies it is clear that *M. a. paratuberculosis* has the potential to survive recommended food preparation techniques and although the surviving numbers may be very small the possibility exists that a chronic additive effect may play a role in Crohn's disease (Millar et al.1996).

Meat Inspection

Current UK legislation does not specifically cite Johne's disease as a condition that could render cattle unfit for human consumption. Only emaciation, oedema and advanced anaemia would lead to condemnation of the carcass (The Fresh Meat (Hygiene and Inspection) Regulations 1995 Schedule10 IX). Meat hygiene staff are required to visually examine the intestines and palpate the mesenteric lymph nodes (Schedule 10 part II of

these Regulations). They are advised to look for intermandibular oedema and "wasting of the buttock muscles" (Gracey and Collins 1992). The earliest lesion is to be found near the ileocaecal valve where the mucous membrane can be four to five times its normal thickness, raised and irregular with longitudinal and transverse corrugations (Gracey, Collins and Huey 1999). Judgment depends on whether the carcass sets (undergoes rigor mortis). Until the recent edition of Meat Hygiene (Gracey et al. 1999), the advice was given to "detain the carcass overnight in a cool dry atmosphere: a number will improve in appearance and dryness, and having value for manufacturing purposes may safely be passed for food" (Gracey et al.1992). Currently in Britain only animals below the age of 30 months may go into the food chain (EC Regulation No 716/96) which would restrict the number of clinical cases of Johne's disease passing through mainstream abattoirs as the average age of clinical disease is 2-5 years (see section 2). However, a recent study in the southwest of England reported that 2% of young cattle (exact age not specified) were IS900 positive in the intestinal lymph node (Cetinkaya et al.1996). Thus the possibility remains that with current meat inspection criteria, the Johne's disease organism, *M. a. paratuberculosis,* may reach the human food chain in subclinically infected cattle.

Aims of this Study

Given the current meat inspection criteria for bovines over six weeks old (Fresh Meat (Hygiene and Inspection) Regulations 1995) the project aimed to assess the efficiency of their use in the abattoir for identifying probable cases of Johne's disease at meat inspection. This would allow detention of the carcass until further diagnosis could be carried out. It was hoped that the study would highlight the most common pathological changes occuring in Johne's disease that could be recognised grossly at post-mortem

examination. The cases selected from animals with clinical disease, seen at the University of Glasgow Veterinary School were considered a worst case scenario and were used to assess the feasibility of these cases meeting the requirements within the guidelines in the regulations and reaching the human food chain.

Cytology, histopathology and PCR were used as a means of detecting the organism in the intestines and in distant sites within the body. Although culture of the organism is the definitive method of diagnosis, the length of time for growth of the organism precluded its use from within the time scale of this project. Nevertheless, material was retained for future culture. The popliteal, prescapular and iliac lymph nodes were of particular interest, as under current abattoir and meat inspection practices in the U.K., organisms within these nodes would remain within the carcass until reaching the cutting plants, where meat is further processed.

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[i]The original description of Johne's disease by Johne and Frothingham is a classical text written in German and has been reviewed by many workers, eg; Chiodini, Van Kruiningen and Merkal 1984a; Jubb, Kennedy and Palmer 1993).

MATERIALS AND METHODS

Cattle with clinical diagnosis of Johne's disease

Selection of cases.

Cattle, purchased by the Division of Farm Animal Medicine and Production, Department of Veterinary Clinical Studies, University of Glasgow, for teaching purposes, and given a clinical diagnosis of Johne's disease, were selected prior to a post-mortem examination. Information regarding ancillary tests was also obtained eg, faecal smear and/or ELISA. An ante-mortem inspection was carried out and the following were noted.

1. age
2. type, eg. beef breed or dairy breed.
3. demeanor
4. presence of diarrhoea
5. hide contamination (see Appendix 1)
6. body condition (SAC Publication No.129)
7. presence of oedema

Necropsy

A gross post-mortem examination was performed following a standard protocol. Samples for cytological smears (see Table 2, page 46) were taken using sterile blades and new, clean, gloves and a clean, fresh working surface used for each sample. The intestines were incised last to minimise the risk of cross contamination. The carcasses were then hung for 24 hours to allow time for "setting" and then assessed by meat inspection criteria. Particular attention was paid to hardening of the fat and mobility of the

antebrachium, rigidity of the skirt (diaphragm) and for evidence of "wetness" of the body cavities.

Microscopic Examinations

Histopathology

Tissue for histopathology was fixed in 10% buffered formal saline and trimmed after 48 hours. The sections were processed through graded alcohols and embedded in paraffin wax; then 4-5μm thick sections were cut and stained with ZN stain for identification of acid-fast bacilli (see Appendix 2) and haematoxylin and eosin (H and E) stains for routine histopathology (see Appendix 3). The sections were studied for clumps of acid-fast bacilli on ZN and for granuloma formation and giant cells on H and E.

Preparation of Cytology Specimens.

Tissues at post mortem were sliced with a sterile scalpel blade and the cut surface impressed on a glass slide. The slides were heat fixed and then stained with ZN.

Examination of Cytological Specimens

The smears were then examined by light microscopy at x100 and under oil immersion for clumps of acid-fast bacilli within cells.

Preparation of PCR Tissue

Collection of PCR Tissue

Tissue samples of variable sizes were taken using sterile blades and gloves and stored at –20°C until further processing.

Preparation of Work Area

The working area was cleaned with 1M HCl before and after each episode at the work bench in a class I microbiological safety cabinet (B.S. 5726).

Preparation of Samples Cell Extraction

Samples were washed to ascertain the level of organisms on the sample surface. 15ml of Millipore molecular grade water was used and the washings collected and centrifuged at 1300rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 200µl of 0.2M NaOH (0.8g NaOH dissolved in 100ml water) and stored at 4°C.

Tissue samples were then seared on their surface and then dissected in a sterile manner so naïve tissue could be harvested from the centre. 1g of tissue was collected where sample size allowed. Where sample sizes were too small they were left intact.

Cell Extraction

1g of tissue was placed in a stomacher bag (Seward Medical Model 80) with 5ml Millipore water and macerated for 5 minutes. 5ml 0.5% w/v NaOH (0.5g NaOH dissolved in100ml water) was added and the fluid fraction of the mixture decanted into a 15ml solvent resistant tube (remaining tissue was discarded for incineration). The mixture was incubated for 30 minutes in a water bath at 56°C.

1ml of xylene was added to the tubes which, were then shaken thoroughly for 2-3 minutes and then allowed to settle. The top (organic) layer was carefully pipetted off, placed into an Eppendorf tube and centrifuged at 1300rpm for 10 minutes. The supernate was decanted. The pellet was resuspended in 500µl 0.2M NaOH, agitated and then

centrifuged again at 13000rpm for 5 minutes. This step was repeated once more and the pellet was finally resuspended in 200µl of 0.2M NaOH and stored at 4°C until required.

Preparation of template for PCR Reaction

Each of the samples were centrifuged at 13000rpm for 10 minutes and the NaOH then pipetted off. 500µl of Millipore-grade water was added, the samples were agitated and then centrifuged again. This step was repeated twice. The samples were then boiled for 15 minutes and allowed to cool before added to the PCR reaction.

PCR Reaction

Preparation of PCR Reaction Mix

The PCR was carried out in a dedicated area, which was cleaned with 1M HCl before and after every use. PCR consumables were stored in a separate –20°C freezer. The following ingredients were used for the reaction:

22.5µl "Reddy load" PCR mix (Advanced Biotechnologies). 22.5µl contains 0.625 units *Taq* DNA polymerase, 37.5mM Tris-HCl (pH8.8 at 25°C), 10nM $(NH_4)SO_4$, 0.75mM $MgCl_2$, 0.015% v/v Tween 20, 0.1mM of each nucleotide dATP, dCTP, dTTP and dGTP.

PCR Primers

The following primers were used for the PCR reaction as described previously by (Moss et al. 1992).

0.5µl of each primer

P90 5' GAA GGG TGT TCG GGG CCG TCG CTT AGG 3' at a dilution of 1:100(1pmol/µl)

P91 5' GGC GTT GAG GTC GAT CGC CCA CGT GAC 3' at a dilution of 1:100 (1pmol/µl) (MWG Biotech).

Final PCR Reaction Mix

1µl of template was added.

The final reaction mix was

0.5µl P90

0.5µl P91

22.5µl "Reddy load" PCR mix

1.0µl DNA template

The mixture was centrifuged for 10 seconds and then placed in a thermocycler. The following protocol was used:

94°C for 30 seconds for denaturation, 55°C for 30 seconds for annealing and 72°C for one minute for extension. This cycle was performed for a total of 30 cycles with a final extension phase of 72°C for 2 minutes.

Visualisation of the PCR Product

PCR product was visualised on a 1.5% agarose gel (1.5g agarose ultrapure (GibcoBRL) dissolved in 100ml TBE* (Tris borate EDTA).

1µl ethidium bromide (10µg/ml) (GibcoBRL) was added. This is a fluorescent dye which intercalates between stacked base pairs of DNA.

*Stock solution of Tris borate EDTA.

5x: 54g Tris base

27.4g boric acid

20ml 0.5M EDTA

Dilute 1:5 from concentrated stock.

10µl of each PCR product was loaded into a well. 10µl of a 1kb DNA ladder (GibcoBRL) was loaded into the first well. The buffer used was the same as that for the gel, Tris borate EDTA.

The gel was run at 100mA for 60 minutes. The final product was visualised under ultraviolet light. A positive result was a band at 400 base pair region in line with the positive control, cervine strain JD88/107 (gift from the Moredun Research Institute).

RESULTS

Gross Inspection

The findings at ante-mortem inspection and gross post-mortem examination are given in Table 1, page 45.

All but one of the animals was aged thirty months or over. Eight of the eleven animals were of Limousin breeding and only one was of a dairy breed (see Table 1, page 45).

Four of the eleven animals had a body condition score of two or above (SAC No. 129) which could be considered fair but only two of these animals had signs of gluteal muscle atrophy and the other two only had intermittent diarrhoea. The remaining seven had chronic diarrhoea and poor musculature.

Only one of the animals had submandibular oedema with oedema of the abomasal folds, two had ascites, one had oedema of the abomasal folds alone and one other animal had oedema of the mesentery.

Five of the eleven animals had enlarged mesenteric lymph nodes (at least twice normal size) (see Figure 3, page 50); all these enlarged lymph nodes were oedematous when cut in longitudinal section from which white watery fluid escaped from the cut surface . Ten of the animals had dilated lymphatic vessels over the serosa and mesentery (see Figure 4, page 51 and Figure 5, page 52). Nine had palpable thickening over the terminal ileum and ten had increased mucosal rugae within the ileum, which could not be flattened out when stretched. One animal (136908) had neither prominent mucosal rugae nor palpable thickening of the terminal ileum but did have prominent dilated lymphatics over the mesentery and the serosa. The terminal ileum of this animal was dilated and fluid filled and the affected region of the gut wall was attenuated.

All the carcasses set within twentyfour hours and were deemed fit to pass meat inspection by two fully qualified meat hygiene inspectors. Incidental findings at post mortem were: 136099 had a small indurated udder with marked enlargement of the right supramammary lymph node that contained gritty calcified foci within the cortex when cut on longitudinal section. Two further animals, 137309 and 136006 had small fibrous, indurated udders.

Results of Cytology

The results of examination of ZN-stained cytological (impression) smears are given in Table 2, page 46.

On cytological examination of specimens from animal136908, no acid-fast bacilli were detected in any tissue tested, although acid-fast organisms were detected in at least one specimen from the other ten cases. Examination of the ileocaecocolic lymph node, ileum, colon and rectum of the other ten cases revealed that, 10/10, 8/10, 9/10 and 9/10 were positive for acid-fast organisms respectively. Five animals were positive in remote sites (distant from the intestinal tract and mesenteric lymph nodes). 136099 was positive in the supramammary and iliac lymph nodes, 136006 was positive in these two lymph nodes and also udder tissue, and both 136176 and 138679 were positive within the hepatic lymph node and 138679 also in the iliac lymph node. Animal 135524 was positive within the iliac (see Figure 6, page 53) and inguinal lymph nodes but many clumps of acid-fast bacilli were observed on sections not associated with cells. Two animals 135524 and 136006 were positive on all sites sampled. 136099 was positive on all cytological specimens except from the ileum and mammary tissue.

In some cases impression smears were not a reliable method as the number of

cells harvested was very sparse and inadequate to be diagnostic .

Results of Histopathological Examination

Results are given in Table 2, page 46 and Table 3, page 47.

None of the eleven animals was positive for acid-fast bacilli in remote sites on histological examination. Nevertheless 7/11 of the animals were positive on both histological and cytological examination of the ileum, 8/11 in the colon, 5/11 in the rectum, 8/11 in both ileocaecocolic and 7/11 in the mesenteric lymph nodes.

Animal 136099 was negative on ZN-stained sections of all histological samples but had prominent granulomas and multinucleate giant cells within many organs when examined by H and E (see Table 2, page 46 and Table 3, page 47). Multinucleate giant cells were mostly confined to intestinal sites and related lymph nodes. Case 136099 and 137309 had multinucleate giant cells in the supramammary lymph nodes which were associated with intensely eosinophilic club-shaped bodies in 136099 (see Figure 7, page 54). Three animals (136176, 136334 and 137309) had multinucleate giant cells within the hepatic lymph node. Animal 136334 also had one within the hepatic parenchyma (see Figure 8, page 55). Case 138679 had prominent histiocytosis of the iliac lymph node but not of the hepatic lymph node. One animal (136573) had a multinucleate giant cell within a retropharyngeal lymph node.

Many of the cases with microgranulomas on H and E were negative when stained with ZN stains (see Table 4, page 48). None of the livers from the selected cases showed macroscopic changes on gross post-mortem examination but on histological examination 6/7 revealed granulomatous lesions, mostly circular foci of macrophages, intermingled with and surrounded by varying numbers of lymphocytes scattered throughout the hepatic

parenchyma (see Figure 9, page 56). Macrophages had abundant foamy cytoplasm with indistinct cell borders forming syncytia described as "symplasma" (Harding 1957). The lesions were avascular and occurred randomly throughout the hepatic parenchyma.

Lesions within the kidney were microscopic and appeared as clusters of predominantly lymphocytes between tubules with no distortion of renal parenchyma.

Intestinal lesions were consistent with classical descriptions. Prominent changes were seen within the lamina propria with invasion by varying amounts of macrophages and lymphocytes, ranging from small focal granulomas to large extensive areas of macrophages forming swathes of symplasma and causing destruction of recognisable anatomy (see Figure 10, page 57). Lesions in some cases were confined to the submucosa.

Polymerase Chain Reaction Results

The results are summarised in Table 5, page 49.

A positive result was assumed when a band was identified at the same level as the positive control, at approximately 400 base pairs.

3/10 animals were positive in the ileocaecocolic lymph node, 4/10 in the colon, and 4/10 were positive in each of the mesenteric lymph nodes, ileum and rectum. In most instances where PCR was positive, the same sample was positive on cytological and histological examination. Only five specimens were positive for PCR but negative for both or either of the microscopic tests (see Table 2). The most significant finding was the prescapular lymph node of animal 136334 (see Figure 11, page 58). The results of histology and cytology with ZN staining were negative but on histological examination of an H and E section of this node, a small granuloma was found (see Table 3).

None of the sample "washings" were IS900 positive by PCR.

Discussion

Until the recent publication of the 10th edition of the Meat Hygiene Inspectors' "bible", Meat Hygiene by Gracey et al., submandibular oedema was said to be one of the earliest signs of Johne's disease and mesenteric lymph nodes were said to be always enlarged (Gracey et al. 1992). Thickening of the terminal ileum and prominent rugae are described. The adherence to ante-mortem inspection regulations should therefore mean that an animal with obvious submandibular oedema would not be permitted to the abattoir for routine slaughter. Nevertheless, such animals could be overlooked and so this animal 138679 was included in the study as a worse case scenario. This animal showed that, even in this extreme case, the carcass would pass subjective meat inspection. Also, whilst one may argue that animals with diarrhoea should not be sent for slaughter, the reality is many animals enter the slaughterhouse with diarrhoea of unknown cause, which is often attributed to parasitic gastroenteritis or stress due to travelling.

The results from the gross post-mortem examination highlighted the range of pathological changes that occur due to Johne's disease. By law, the meat hygiene inspectors are required to palpate the mesenteric lymph nodes and visually examine the intestines (Fresh Meat (Hygiene and Inspection) Regulations 1995). Gracey and Collins (1992) and Gracey et al. (1999) advises that mesenteric lymph node enlargement is typical. In this study we found that less than half of the cases (45%) showed this at post-mortem. More consistent findings were palpable thickening of the terminal ileum and increased thickness of the mucosal rugae (82% and 91% respectively).

Following complete examination, animal 136908 was considered not to be a case of Johne's disease because all diagnostic tests were negative for *M. a. paratuberculosis*.

Gross post-mortem findings were not typical of Johne's disease and the presence of the ileal dilation resulted in the diagnosis of ileal dilation of unknown aetiology.

At necropsy examination of 136908, mesenteric lymph nodes were enlarged and lymphatic vessels dilated and prominent over the mesentery and the serosa. The ileal wall was not thickened but attenuated and there was no "cobblestoning" of the mucosal rugae. Although faecal smear was positive, only one bacilli was identified. This was most likely a saprophytic organism, which is common in the faeces of cattle (Chiodini et al. 1984a).

All of the ten confirmed cases of Johne's disease had thickening and "cobblestoning" of the terminal ileum. It is of interest to note that these features would not be obvious to meat hygiene inspectors at routine meat inspection, as the meat inspector has to neither touch or incise the intestines.

All of the ten carcasses set, which is one of the major criteria for passing meat inspection. The carcasses would be considered "fit" for human consumption, however due to their poor conformation, they would probably have been downgraded and used for manufacturing purposes. This is of particular concern as it is this type of meat that will be used for the production of sausage meat, mince and precooked products. The reliability of heat killing of *M. avium-intracellulare* organisms has been doubted when current methods of pasteurisation which are aimed at killing most pathogens particularly *Salmonella spp.* are used (see Introduction). It is thought that the internal temperature of the products may not reach the killing range (over 60^0C for 5 minutes) for long enough if at all (Merkal et al. 1979).

Some of the results were of particular interest. The reason for 136099 being negative on ZN-stained sections of all histological sections remains unclear. Cytology smears were positive, even in remote sites. The possibility that the slides had been

contaminated was considered. However, faecal contaminants were not identified on the slides and bacilli were identified within cells, indicating that infection and phagocytosis of organisms had occurred in life. Mycobacteria are known to occur in foci throughout the length of the intestine and numbers are likely to be small in extra-intestinal sites so in some circumstances it would be quite feasible to miss granulomas containing bacilli when collecting samples. However this explanation is less likely since all sections of samples were negative. Organisms in positive control material stained well, indicating that the staining procedure was adequate. This animal did have discrete granulomas in many organs throughout the body (see Table 3) and highlighted the need to take serial sections to maximise the chance of identifying organisms. The results from this animal could, however, be explained by the presence of mycobacterial organisms in the form of spheroplasts (see Introduction) and it may be that organisms with incomplete cell walls lose their acid-fast properties during the process of passing through graded alcohols. Paucibacillary enteritis which has been described in sheep (Clarke and Little 1996) is thought to occur in cattle. Cell wall deficient, spheroplast like organisms have been detected in paucibacillary intestinal lesions in cattle with subclinical Johne's disease (Condron et al. 1994). Section of the right supramammary lymph node revealed eosinophilic club-shaped bodies (Splendor-Hoeppli bodies) surrounded by a mixed population of macrophages and many multinucleate giant cells (see Figure 7). This could possibly be *Actinobacillus* mastitis. Case 137309 had multinucleate giant cells within the supramammary lymph node. The sections showed irregular aggregates of mononuclear inflammatory cells with fibrosis but did not have the distinctive Splendor-Hoeppli bodies (Jubb et al. 1993). Animal 136006 was positive on cytological examination of all samples but histological

examination was positive only in intestinal sites and associated lymph nodes. On H and E sections, histiocytosis was found in the iliac lymph node and within the supramammary lymph nodes.

The reason for the animals being positive in the iliac lymph node may be because the iliac lymph node drains the anorectal lymph nodes that directly drain the anus, rectum and descending colon (Schummer, Wilkens,Vollmerhaus and Habermehl 1981). The anorectal lymph nodes are found embedded within the subserosa of these organs. The positive hepatic lymph node cytology and the finding of multinucleate giant cells within the hepatic lymph nodes of three animals can be explained by the fact that these lymph nodes are those draining the mesentery and the gut (Schummer et al. 1981).

Most of the animals (10/11) had organs containing microgranulomas and many carcass lymph nodes had evidence of histiocytosis (see Tables 3 and 5) though no acid-fast bacilli were seen on ZN-stained section. This is consistent with a previous report of a case of a 2.5 year old cow where indirect immunoperoxidase staining was used to identify the presence of paratuberculosis antigens within the lesions (Hines et al. 1983). In experimentally and naturally infected sheep and goats, small numbers of acid-fast bacilli can be identified within remote sites of the body (Harding 1957; Nakamatsu and Fujimoto1968; Rajya and Singh1961). Experimental infection in goats revealed there was a high correlation between the presence of lesions and the presence of *M. a. paratuberculosis* isolated in culture (Harding 1957) but it must be borne in mind that granulomatous formation within the body is a nonspecific inflammatory reaction by the immune system and culture would be required to confirm the presence of the organism.

Node-negative, gut-positive results could possibly occur and could be attributed

to very localised intestinal infections but may also be attributed to the difficulty in identifying the organism in some lesions where numbers of the organism are low.

To date IS900 is thought to be specific for *M. a. paratuberculosis* (Sockett et al. 1992). Thus the finding of a positive result from the prescapular lymph node of 136334 is significant as this suggests that a generalised infection of the carcass has occurred. This is of public health significance as carcass lymph nodes remain with the carcass until reaching the cutting plant where they would be trimmed off and the possibility of contamination of meat and of the workers hands is therefore possible. With the possibility that systemic infection comes about due to a bacteraemia, this finding raises concern about the degree to which the afferent muscle groups could be affected.

The sensitivity of the PCR was questioned when in 15 sites IS900 was not detected but cytology and histology were positive and in 31 sites where IS900 was not detected but either of the other two tests were positive. This correlates with the findings of Sockett et al. (1992) who found the PCR method to be the least sensitive method when compared to radiomimetric and conventional culture, when tested on faeces. Lack of sensitivity was thought to be related to PCR inhibitors and also that used for extraction of the organisms from the tissue and the method used for extracting DNA from the cells. A method has been described where the PCR can detect as little as 5-10 mycobacteria/gram of sample when radiolabelled DNA visualisation methods are used (Challans, Stevenson, Reid and Sharp 1994). Comparison of results (see Tables 3 and 4) reveals that many of the samples negative by PCR contain granulomatous lesions and would suggest that culture and a more successful method for DNA extraction may find more organisms present within the body. More recently, the specificity of IS900 came into question again

with the finding of IS900 positive isolates during surveillance in Western Australia and Victoria (Cousins, Whittington, Masters, Marsh, Evans and Kluver 1999). Four isolates were found to be IS900 positive but Mycobactin independent. Restriction digests were not consistent with *M. a. paratuberculosis*. Sequence differences were detected among the strains within the amplified IS900 PCR product resulting in homologies between 72% and 81% with *M. a. paratuberculosis*. Therefore, further definition of IS900 positive samples using restriction enzymes is advised (Cousins et al. 1999).

	Age/breed	BCS	smear	diarrhoea	wasting	carcass set	oedema	MLN	lymphatics	rugae	ileum	LN oedema
135816	5y/limx	3	-	+/-	-	+	-	-	+	+	+	+
136099	3y/gall	2.5	+	+	+	+	mesentery	+	+	+	+	+
136006	3y/limx	1	+	+	+	+	-	-	+	+	+	-
135524	5y/lim	1.5	+	+	+	+	-	-	+	+	+	-
136176	6y/friesian	2	+	+	+	+	-	-	+	+	+	-
136908	1y/simx	1.5	+	+	+	+	-	+	+	-	-	+
136334	5y/lim	1	+	+	+	+	ascites	+	+	+	-	+
136573	6y/limx	1.5	-	+	+	+	-	+	+	+	+	+
137309	8y/limx	1.5	+	+	+	+	ascites	-	+	+	+	+
138560	4y/lim	3	+	+/-	-	+	abomasum	+	+	+	+	+
138679	8y/lim	1.5	+	+	+	not jaw	Jaw& abomasum	-	-	+	+	-

Table 1. Gross post-mortem findings of clinical cases.

Abbreviations. BCS = Body Condition Score, MLN = Mesenteric lymph node, + = enlarged, - = not enlarged; lymphatics refers to dilation of mesenteric lymphatics + = presence , - = absence; ileum refers to thickening of terminal ileum + = presence, - = absence; rugae refers to "cobblestoning" of the ileum + = presence, - = absence.

Breeds: Lim = Limousin, Gall = Galloway, Sim = Simmental, X = hybrid.

+/- = intermittent diarrhoea.

	135816	136099	136006	135524	136176	136908	136334	136573	137309	138560	138679
IC*	+ + -	+ - +	+ + +	+ + +	+ + -	- - -	+ + -	+ + np	+ + -	+ + -	+ - -
SM*	- - -	+ - -	+ - -	ns	- - -	ns	- - -	- - np	- - -	ns	ns
Iliac*	- - -	+ - -	+ - -	+ - -	- - -	- - -	- - -	- - np	- - -	- - -	+ - -
Popliteal*	ns	ns	ns	ns	- - -	- - -	- - -	- - np	- - -	- - -	- - -
Prescap*	ns	ns	ns	ns	- - -	- - -	- - +	- - np	- - -	- - -	- - -
Hepatic*	ns	ns	ns	ns	+ - -	- - -	- - -	- - np	- - -	- - -	+ - -
Bronch*	ns	ns	ns	ns	- - -	- - -	- - -	- - np	- - -	- - -	- - -
Retro*	ns	ns	ns	ns	- - -	ns	- - -	- - np	ns	- - -	- - -
Tonsil*	ns	ns	ns	ns	ns	ns	- - -	- - np	ns	- - -	- - -
Mes*	+ + -	+ - -	+ + +	+ + +	+ - -	- - -	+ + +	+ + np	+ + +	+ + -	ns
Lumbar*	ns	ns	ns	ns	- - -	- - -	- - -	- - np	- - -	- - -	- - -
Ileum	+ + -	- - -	+ + +	+ + +	+ + +	- - -	+ - +	+ + np	- - +	+ + -	+ + -
Colon	- + -	+ - -	+ + +	+ + +	+ + -	- - -	+ + +	+ + np	+ + -	+ + -	+ + +
Rectum	+ + -	+ - -	+ + +	+ + +	+ - -	- - -	+ - +	+ - np	+ + -	+ + +	- - -
Lung	ns	ns	ns	ns	- - -	- - -	- - -	ns np	- - -	nd - -	nd - -
Liver	ns	ns	ns	ns	- - -	- - -	- - -	nd - np	nd - -	- - -	- - -
Kidney	ns	ns	ns	ns	- - -	- - -	- - -	nd - np	nd - -	- - -	nd - -
Mammary	- - -	- - -	+ - -	ns	- - -	ns	- - -	- - np	nd - -	ns	- - -
Inguinal*	ns	ns	ns	+ - -	ns	ns	ns	ns np	ns	- - -	- - -

Table 2. Summary of Laboratory Results. Cytology, Histology and PCR respectively in each column.
Cytology and histology: + = presence, - = absence of acid-fast bacilli, PCR + = positive, - = negative for IS900.
Abbreviations: IC =Ileocaecocolic, SM = Supramammary, Prescap = Prescapular, Bronch = Bronchial, Retro = Retropharyngeal, Mes = Mesenteric, np = not performed, nd = not diagnostic, ns = not sampled.
* = Lymph node.

	135816	136099	136006	135524	136176	136908	136334	136573	137309	138560	138679
IC*	+++//	+++///	+++///	++	++	++	+++//	+++///	+++/	++	0
SM*	++	+++///	+++	ns	+++	Ns	+	+	++//	ns	ns
Iliac*	0	+	+	++	+++	0	++	+++	+N	+	++
Popliteal*	ns	ns	ns	ns	0	++	-	++	+++	+	+
Prescap*	ns	ns	ns	ns	+	++	+	++	+	+	0
Hepatic*	ns	ns	ns	ns	++/	0	++//	+++	+/	+	0
Bronchial*	ns	ns	ns	ns	++	+	++	+++	0	+	+
Retro *	ns	ns	ns	ns	++	ns	+++	++/	ns	-	+
Tonsil*	ns	ns	ns	ns	ns	ns	+	0	ns	+	-
Mes*	++/	++//	+++///	++	++//	+	+++///	+++	+++///	++	ns
Lumbar *	ns	ns	ns	ns	++	+	++	+	+N	0	++
Ileum	+++/	+++///	+++///	+++///	+++///	0	++//	+++///	-	+++/	++//
Colon	++/	+++///	+++///	+++//	+++/	+	+++///	+++///	++//	++	+++
Rectum	++	+++///	+++///	+	-	+	+++///	+	+++//	++	++
Lung	ns	ns	ns	ns	-	-	+	ns	+	-	-
Liver	ns	ns	ns	ns	-	+	++/	++	+	++	+
Kidneys	ns	ns	ns	ns	0	0	+	-	+	-	+
Mammary	+	0	+++		-	ns	0	+	++	ns	-
Inguinal *	ns	ns	ns	0	ns	ns	ns	ns	ns	+	+

Table 3. Microscopic Examination of H and E stains. + =Granulomas present, / = multinucleate giant cells present.
+ = small accumulation of macrophages surrounded by mononuclear cells
++ = accumulation of macrophages with mononuclear cells but no disruption of tissue architecture.
+++ = extensive accumulation of macrophages and mononuclear cells with disruption of organ architecture.
/ = One multinucleate giant cell present per low power field.
// = Two to four multinucleate giant cells per low power field.
///= More than four multinucleate giant cells per low power field.
N = Neutrophils present, ns = not sampled, 0 = no granulomatous lesions present.
Abbreviations: SM = Supramammary, IC = Ileocaecocolic, Prescap = Prescapular, Retro = Retropharyngeal, Mes = Mesenteric.
* = Lymph node.

	135816	136099	136006	135524	136176	136908	136334	136573	137309	138560	138679
IC*		+									
SM*	+	+					+	+	+		
Iliac*		+	+		+		+	+	+	+	+
Popliteal*								+	+	+	+
Prescap*					+		+	+	+	+	
Hepatic*					+		+	+	+		
Bronch*					+		+	+		+	+
Retro*					+		+	+		+	+
Tonsil*							+				
Mes*		+			+						
Lumbar*					+		+		+		+
Ileum		+									
Colon		+									
Rectum		+					+	+			
Lung							+		+		
Liver							+	+	+	+	+
Kidneys							+		+		+
Mammary	+	+						+	+		
Inguinal*							+			+	+

Table 4. Samples with granuloma formation or histiocytosis but ZN stain negative on histology sections. These are indicated with +.
Abbreviations: IC = Ileocaecocolic, SM = Supramammary, Prescap = Presapular, Retro = Retropharyngeal,, Mes = Mesenteric.
* = Lymph node.

	135816	136099	136066	135524	136176	136908	136334	136573	137309	138560	138679
IC*	-	+	+	+	-	-	-	na	-	-	-
SM*	-	-	-	ns	-	ns	-	na	-	ns	ns
Iliac*	-	-	-	-	-	-	-	na	-	-	-
Popliteal*	ns	ns	ns	ns	-	-	-	na	-	-	-
Prescap*	ns	ns	ns	ns	-	-	+	na	-	-	-
Hepatic*	ns	ns	ns	ns	-	-	-	na	-	-	-
Broncial*	ns	ns	ns	ns	-	-	-	na	-	-	-
Retro*	ns	ns	ns	ns	-	-	-	na	-	-	-
Tonsil*	ns	ns	ns	ns	-	-	-	na	-	-	-
Mes*	-	-	+	+	-	-	+	na	+	-	ns
Lumbar*	ns	ns	ns	ns	-	-	-	na	-	-	-
Ileum	-	-	+	+	+	-	+	na	+	-	-
Colon	-	-	+	+	-	-	+	na	-	-	+
Rectum	-	-	+	+	-	-	+	na	-	+	-
Lung	ns	ns	ns	ns	-	-	-	na	-	-	-
Liver	ns	ns	ns	ns	-	-	-	na	-	-	-
Kidney	ns	ns	ns	ns	-	-	-	na	-	-	-
Mammary	-	-	-	ns	-	ns	-	na	-	ns	-
Inguinal*	ns	ns	ns	-	ns	ns	ns	na	ns	-	-

Table 5. PCR results.
Abbreviations: + = positive for IS 900, - = negative for IS 900, ns = not sampled, na = not available.
IC = ileocaecocolic, SM = Supramammary, Prescap= Prescapular, Retro = Retropharyngeal, Mes = Mesenteric.
* = Lymph node.

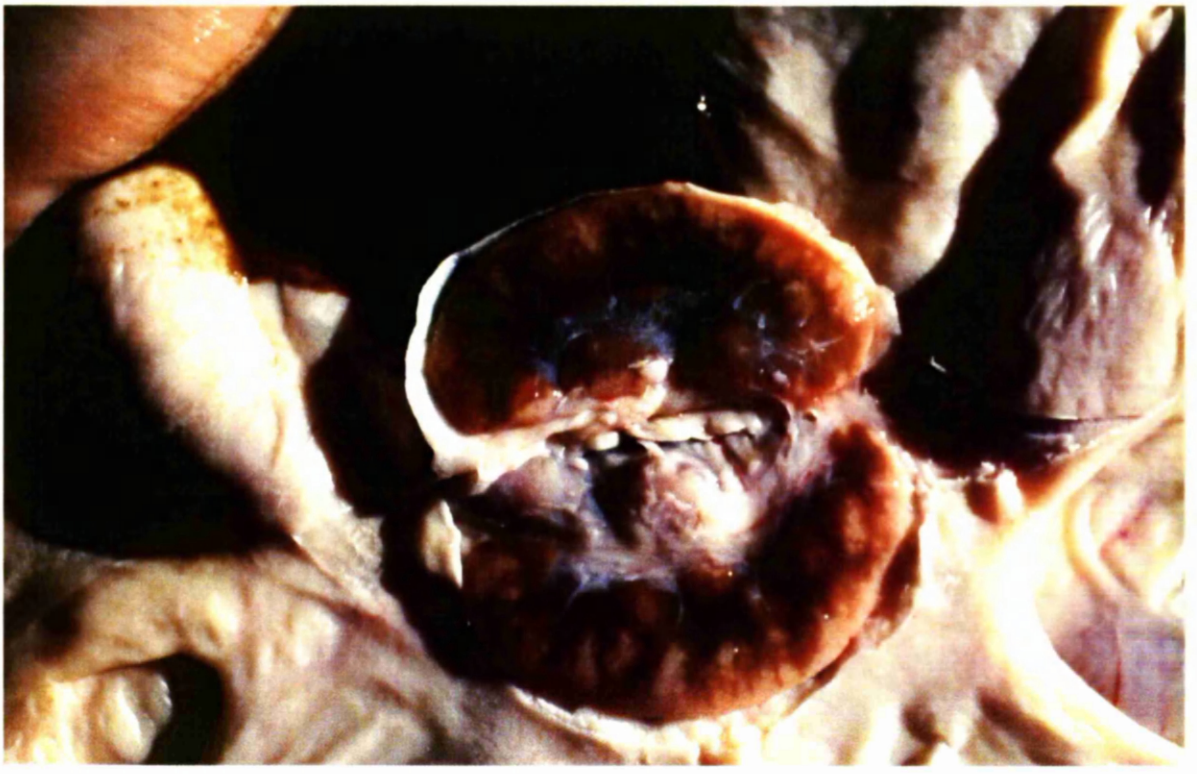

Figure 3. Case 136099, a transverse section through an enlarged and fleshy mesenteric lymph node.

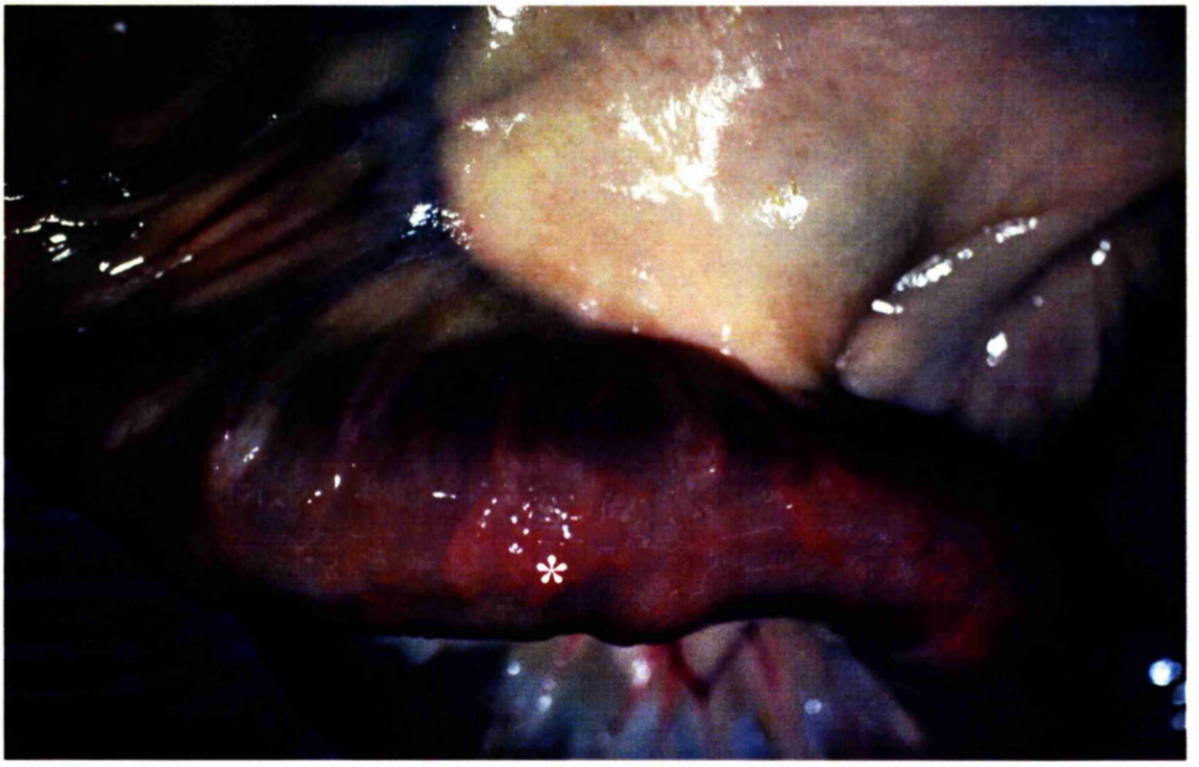

Figure 4. Case 136099, a gross section of terminal ileum demonstrating dilated lymphatics with hyperaemia (asterisk).

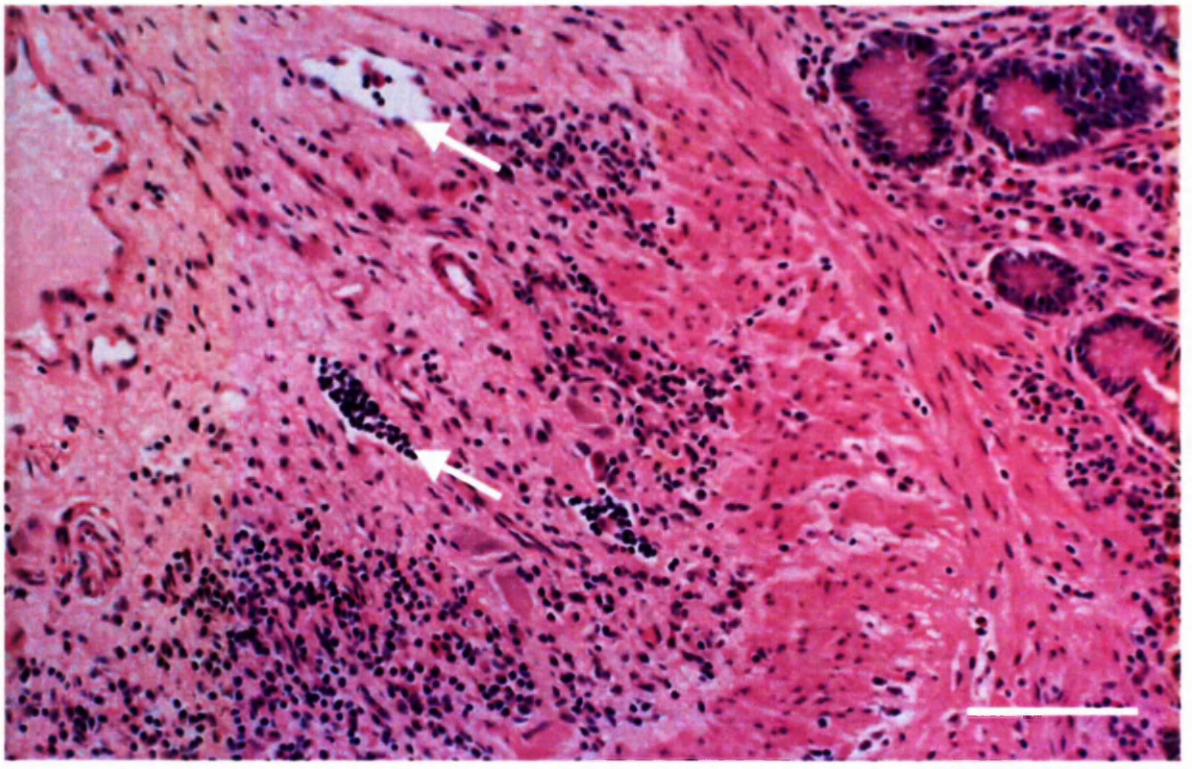

Figure 5. Case 136099, a paraffin wax-embedded section of terminal ileum (Figure 4), stained with H and E. Lymphatic vessels within the submucosa are dilated and filled with mononuclear cells (arrows).

(bar = 100μm)

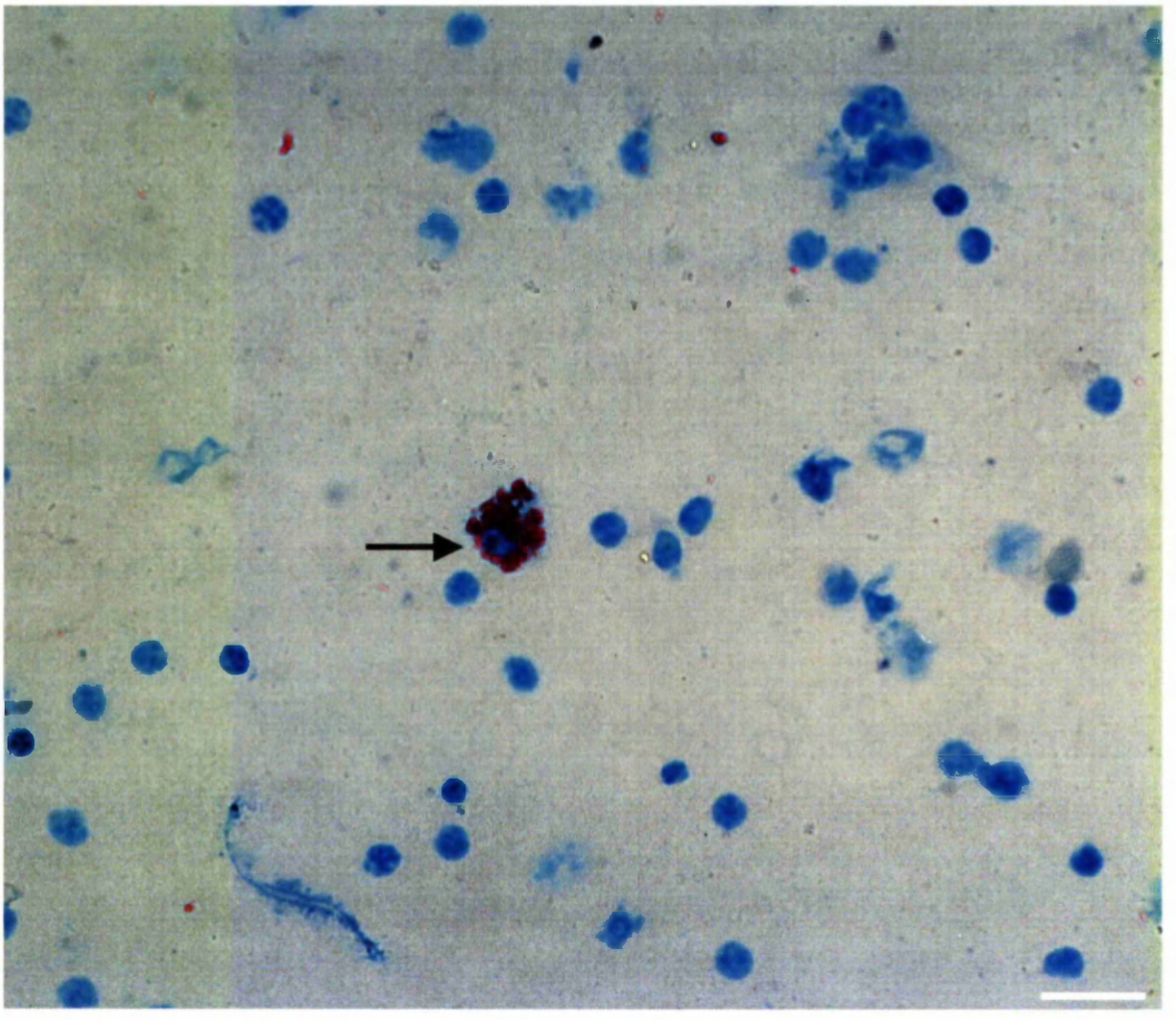

Figure 6. Case 135524, a cytological smear of Iliac lymph node stained with ZN. A macrophage can be seen containing intracytoplasmic acid-fast bacilli that obscure the nucleus (arrow).

(bar = 2μm)

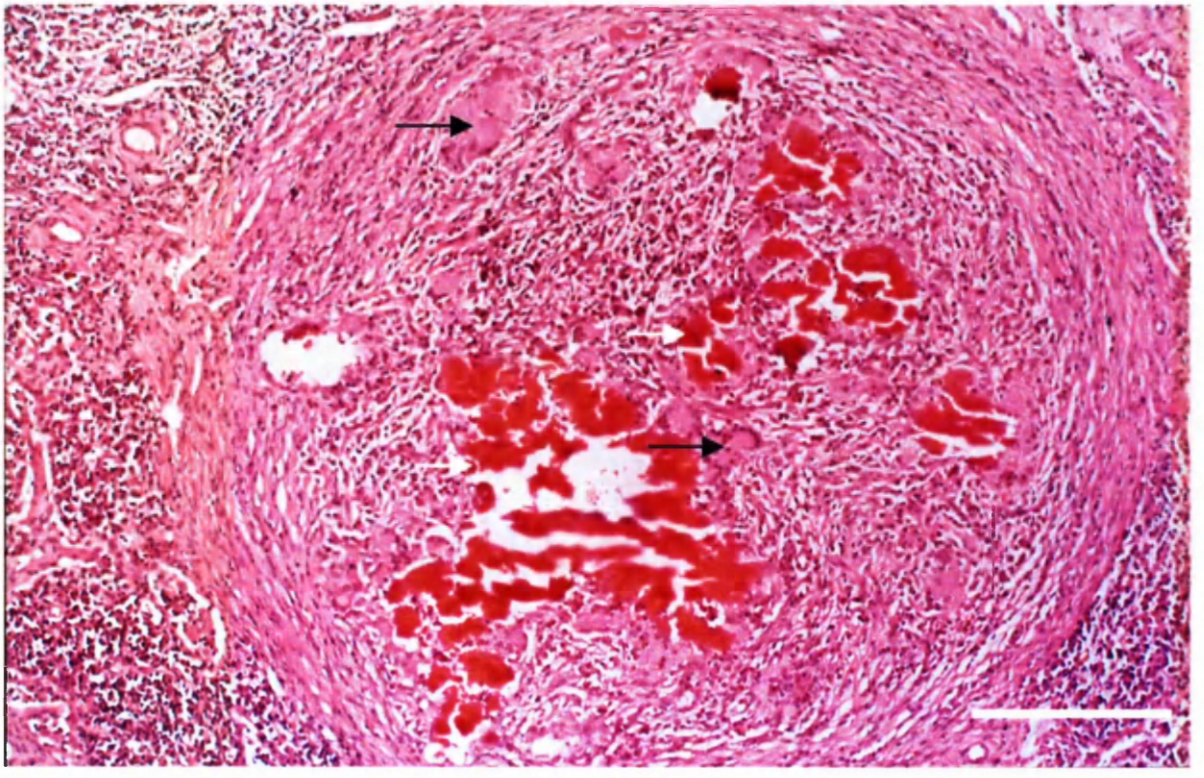

Figure 7. Case 136099, a paraffin wax-embedded section of supramammary lymph node stained with H and E. Club shaped eosinophilic bodies, Splendor-Hoeppli bodies (white arrows), are surrounded by a large population of mononuclear cells. Many multinucleate giant cells can be seen (black arrows).

(bar=200μm).

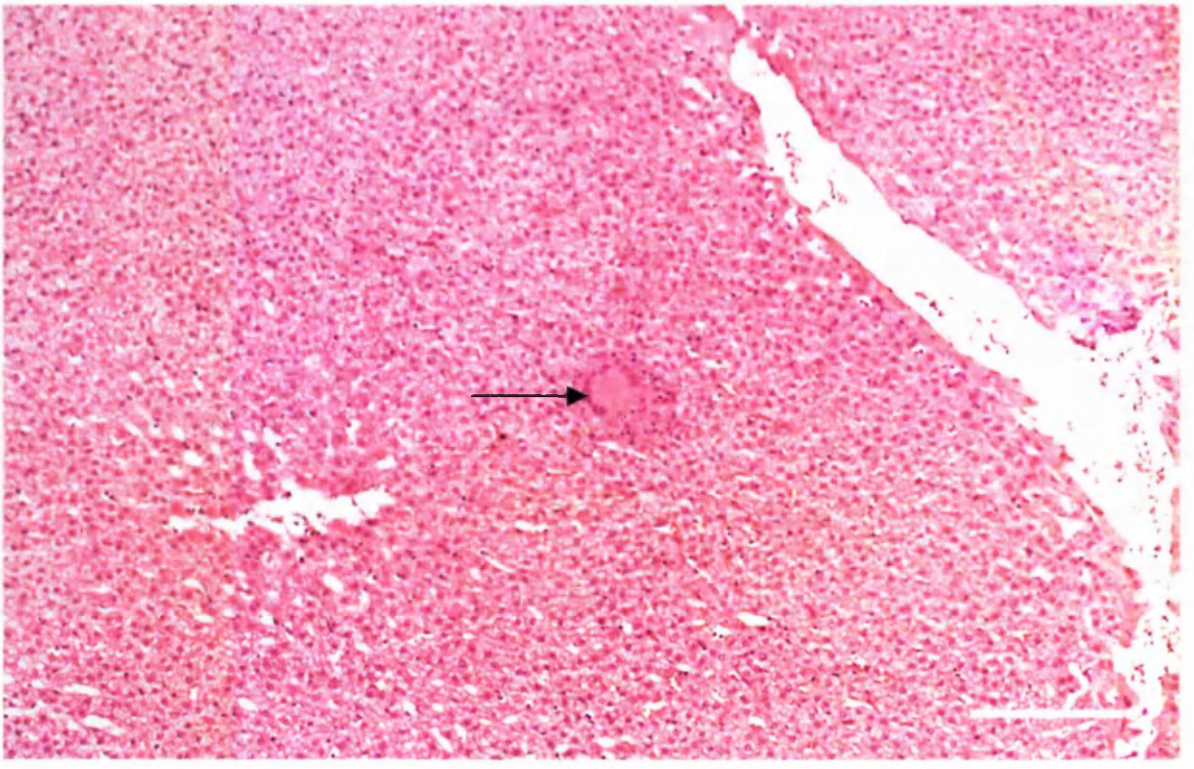

Figure 8. Case 136334, a paraffin wax-embedded section of liver stained with H and E. A solitary multinucleate giant cell can be seen within the hepatic parenchyma surrounded by a small number of lymphocytes (arrow). (bar=200μm)

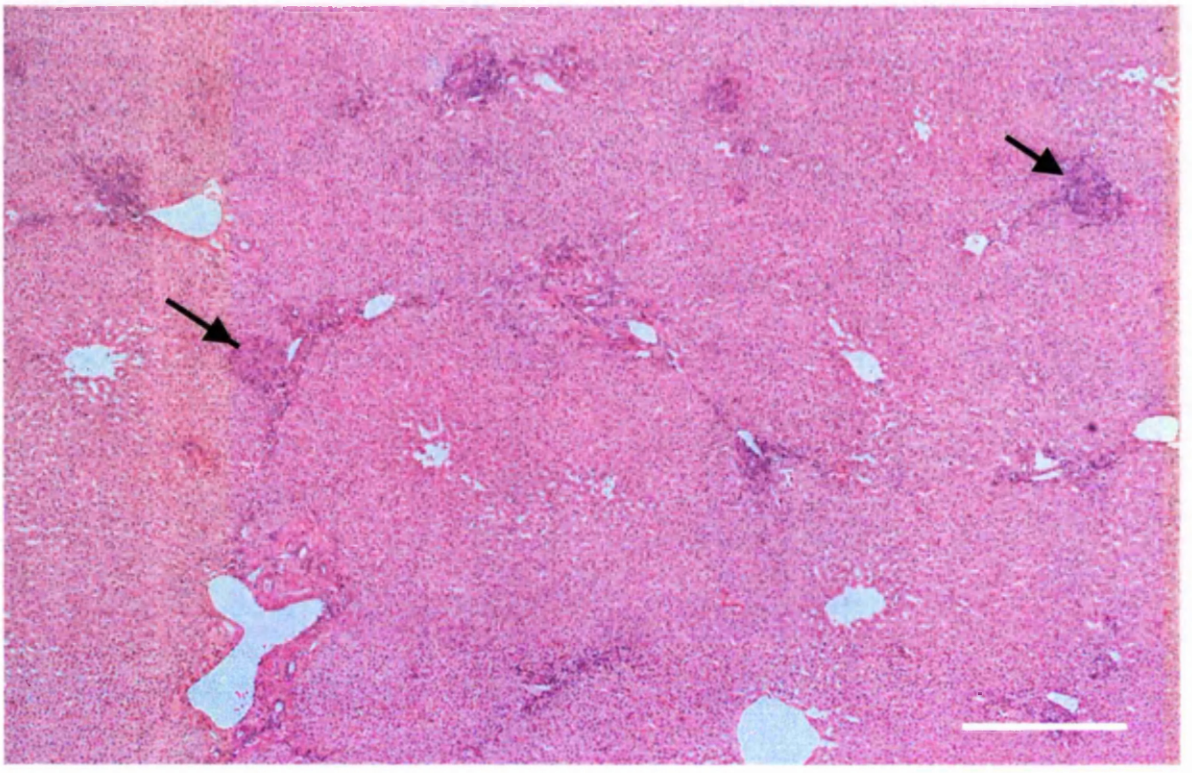

Figure 9. Case 136334, a paraffin wax-embedded section of liver stained with H and E. Frequent irregular foci of mononuclear cells can be seen investing the hepatic parenchyma (arrows).

(bar= 500μm)

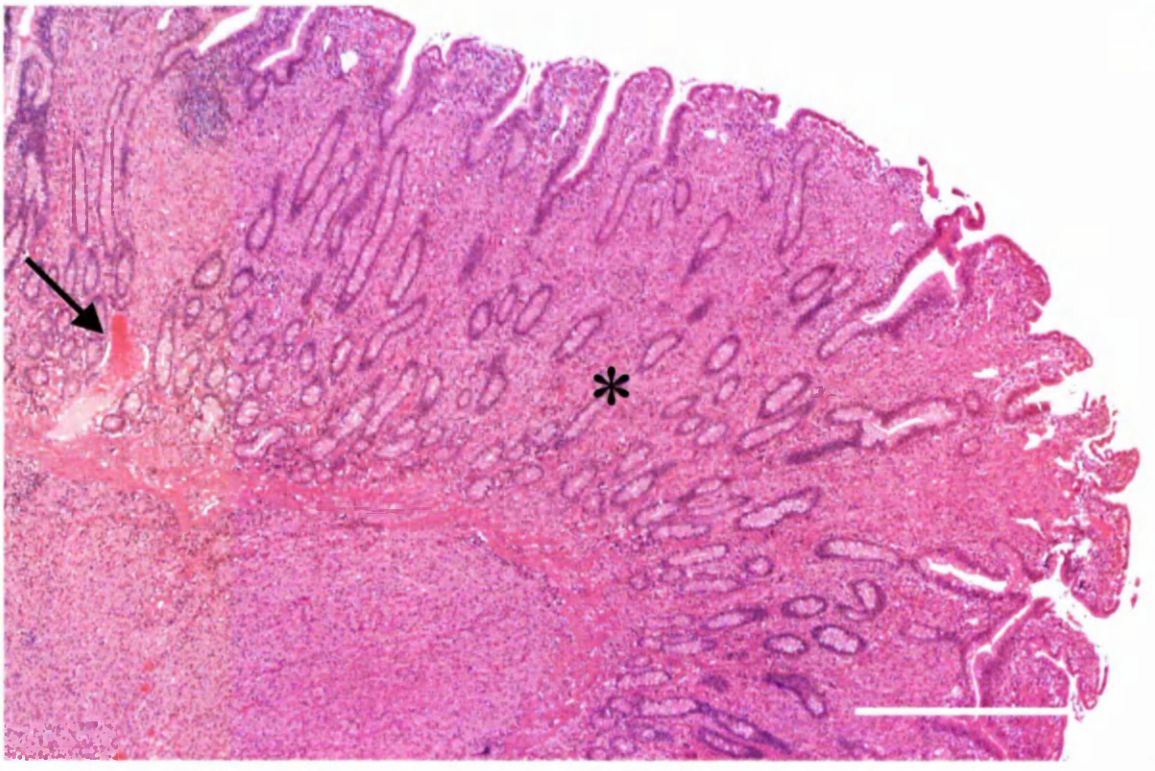

Figure 10. Case 136006, a paraffin wax-embedded section of terminal ileum stained with H and E. Villi are blunt and fused with distortion of villus crypts. A distended capillary can be seen dilated and filled with eosinophilic material (arrow). The lamina propria is infiltrated by abundant mononuclear cells (asterisk). The infiltrate extends down into the submucosa.

(bar=500μm)

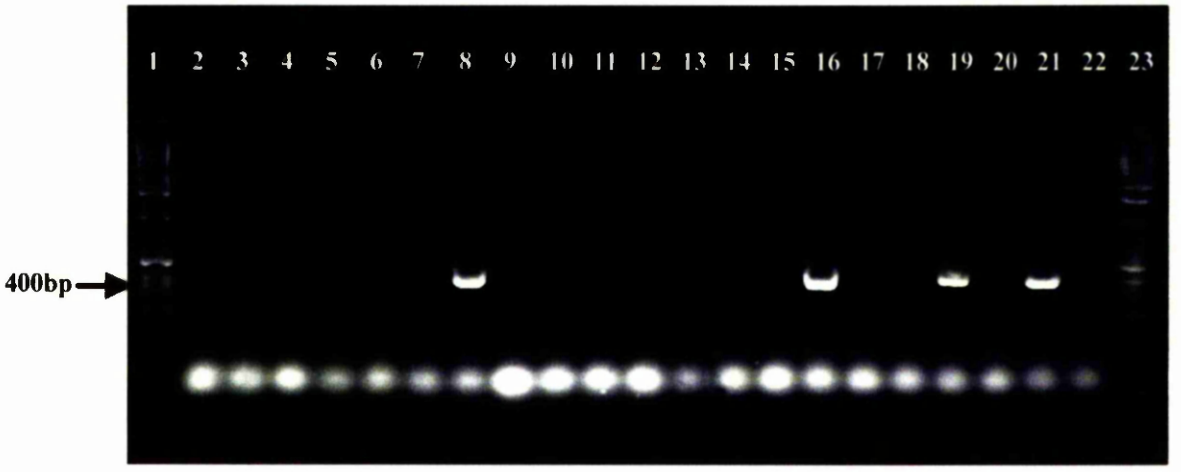

Figure 11. PCR analysis of total DNA extracted from tissue samples from case 136334 using primers designed to detect the IS900 insertion element (413bp product).

Lane no.	DNA from tissue sample
1	100bp size marker
2	popliteal lymph node
3	liver tissue
4	udder tissue
5	lumbar lymph node
6	rectum tissue
7	left kidney tissue
8	mesenteric lymph node
9	lung tissue
10	prescapular lymph node
11	supermammary lymph node
12	iliac lymph node
13	hepatic lymph node

Lane no.	DNA from tissue sample
14	tonsil
15	retropharyngeal lymph node
16	colonic tissue
17	right kidney tissue
18	bronchial lymph node
19	ileal tissue
20	splenic tissue
21	positive control*
22	negative control (no target DNA)
23	100bp size marker

Lanes 6, 8, 10, 16 and 19 contain PCR products of the appropriate size indicating the presence of *M. a. paratuberculosis* genomic DNA within these tissue samples.

* = cervine *M. a. paratuberculosis* DNA.

STUDY OF CATTLE AT ABATTOIR

Introduction

The study was conducted at an over thirty month scheme abattoir (OTMS) (a slaughterhouse only killing animals over thirty months) to investigate the ease at which more invasive examination of the carcass could be carried out within the commercial process.

Samples were harvested from cattle at Kilmarnock abattoir. Cattle at this abattoir were selected as they would all be older than 2.5 years and so were more likely to have clinical signs of Johne's disease. Although at present these animals do not go for human consumption due to EC Regulation 716/96, they did so up until July 1996 and still do in other countries at the present time. Therefore it was of interest to investigate how easy these animals would be to select in the slaughterhouse environment.

Materials and Methods

Selection of Cases

Cattle were selected on the basis of poor body condition and signs of diarrhoea. Body condition scores were not given, as the animals were not accessible for palpation. All cases selected were of clean livestock policy grade 2 (see Appendix 1) so they would have been declared suitable for slaughter in an under 30 month abattoir. Only one visit was made to the OTMS abattoir.

Sample Collection

The alimentary tracts were assessed in the "gut room" for thickening of the terminal ileum, mucosal "cobblestoning" and dilated mesenteric lymphatic vessels.

Samples for cytological and histological examination and also PCR were taken with sterile blades and clean gloves for each sample (as for clinical cases, see chapter 2). Each sample was stored for transportation in a separate clean plastic bag, which was then sealed.

Transportation of Samples

Samples collected were stored in a blue crate designated for Specified Bovine Material (S.B.M.). The material was weighed and recorded on an OTMS 26 form in accordance with the current legislation. The samples were then transported directly to University of Glasgow Veterinary School where they were processed as previously described (see Chapter 2).

Results

All cases selected were of poor body condition and showed some degree of gluteal atrophy. Cow no. 1 showed enlargement of the mesenteric lymph nodes and dilated lymphatic vessels over the mesentery and the serosa (see Table 6, page 62) but there was no palpable thickening of the terminal ileum or "cobblestoning" of the ileal mucosa.

All laboratory tests were negative (see Table 7, page 63) and so all cases were considered not to be cases of Johne's disease. Although all sections were negative with ZN stains, some lymph nodes (supramammary, ileocaecocolic, and iliac lymph nodes) and tonsils showed some histiocytosis.

Cow No.	Age	Breed	BC	CLP	Diarrhoea	Wasting	Mes	Lymphatics	Rugae	S.I. thickening
1	9y	Ayrshire	poor	2	+	+	+	+	-	-
2	8y	Friesian	poor	2	+	+	-	-	-	-
3	7y	Friesian	poor	2	+	+	-	-	-	-
4	5y	Friesian	poor	2	+	+	-	-	-	-
5	9y	Friesian	poor	2	+	+	-	-	-	-

Table 6. Results of Gross post-mortem examination from Kilmarnock Slaughterhouse.
Abbreviations: CLP= Clean Livestock Policy (see Appendix 1), Mes = Mesenteric lymph node, + = enlarged, - = normal, Lymphatics, = = dilation present, - = dilation absent, S.I.= Small Intestine, B.C.= Body condition, Rugae + = presence, - = absence of cobblestoning, diarrhoea + = present, - = absent.

Cow No.	Ileum	IC*	SM*	Iliac*	Popliteal*	Prescapular*	Retro*	Tonsil*
1	- - -	- - -	- -° -	- - -	- - -	- - -	- - -	- - -
2	- - -	- - -	- -° -	- -° -	- - -	- - -	- - -	- -° -
3	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
4	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
5	- - -	- -° -	- - -	- -° -	- - -	- - -	- - -	- - -

Table7. Summary of Laboratory Results. Cytology, Histology and PCR respectively in each column.
Abbreviations: IC = Ileocaecocolic, SM = Supramammary, Retro = Retropharyngeal.
* = Lymph node.
Cytology and histology, - = acid-fast bacilli absent, + = acid-fast bacilli present.
PCR, - = IS900 negative, + = IS900 positive.
° = histiocytosis present.

Discussion

All animals chosen at the abattoir were considered negative for Johne's disease, as all tests conducted were negative. Examination of histological sections revealed histiocytosis of several lymph nodes within the carcasses (see Table 7). This highlights the fact that granulomatous formation can have many aetiologies and alone cannot be considered supportive of a diagnosis of Johne's disease. Nevertheless, when correlated with positive laboratory tests, such findings may be suggestive of further spread of the organism within the carcass.

All the animals were selected on the basis of having diarhoea, highlighting the occurrence of diarrhoeic cattle entering the slaughterline (see Table 6). In this slaughterhall, a more thorough inspection of the intestinal tract was possible within the time constraints of the slaughterline and could be conducted in the gut room, separate from the carcass.

The results from the Kilmarnock OTMS abattoir were disappointing, but were in keeping with the low prevalence of Johne's disease in the cattle population (see Introduction). Sampling five animals is inadequate but time was limited (only one abattoir visit was possible in the time course of the project). Thus the project acted as a pilot study to investigate the possibility of carcasses affected with Johne's disease passing current meat inspection legislation and entering the food chain and how easily these cattle could be selected on the slaughterline.

Furthermore, this study proved that the intestines can be incised and inspected in the gut room, with minimal potential for contamination of the slaughterline and can be

executed in approximately 1-2 minutes. This would require one meat inspection station to be within the gut room. The meat hygiene inspector on this station would not be able to exchange stations unless changing overalls and washing between stations. The cost effectiveness of this station would have to be assessed as the meat hygiene inspector would be restricted to this site alone whereas currently, in times where staffing shortages are problematic, the gut inspection can be done by the inspector examining the heads.

This study has highlighted the feasibility of examining the guts more thoroughly in the abattoir without greatly increasing the risk of contamination of the slaughterline nor causing significant delay in the speed of the process.

At present the expense of such an exercise may be disproportionate to the risk of Johne's disease affected carcasses contributing to Crohn's disease in humans. But if scientific evidence continues to add weight to a mycobacterial aetiology for Crohn's disease, it would be pertinent to look for these carcasses and condemn them.

GENERAL DISCUSSION

Discussion

From this study (Chapter 4) we have highlighted the fact that many cattle are admitted to the abattoir with diarrhoea of unknown origin which is ascribed to stress due to travel, handling and the strange surroundings or parasitism. Some of these animals may be infected with *M. a. paratuberculosis*. An abattoir study carried out on cattle in the southwest of England found that 3.5% of older cattle and 2.0% of young cattle were positive for the IS900 insertion sequence, diagnostic for Johne's disease, when the ileocaecocolic lymph node was tested. All these animals were therefore considered subclinical carriers of the bacteria (Cetinkaya et al.1996).

The argument that Johne's disease is a disease of cattle of 2 years old and older and, with the current OTM legislation these animals are likely to be excluded from the food chain, does not take into account that animals may be infected in utero (Alexejeff-Goloff 1929; Pearson and McClelland 1955; Lawrence 1956; Doyle 1958; Kopecky, Larsen and Merkal 1967; Sietz et al. 1989) and are more susceptible to infection and development of the disease when infected early in life, under 6 months of age (Doyle 1953 and 1958). Investigation of the necropsy database (1988-2000) at University of Glasgow Veterinary School, revealed that 65 cattle were given the diagnosis of Johne's disease. Twelve of these animals (19%) were under 30 months of age. Furthermore the disease may be subclinical for many years and the incidence of subclinical disease is thought to be 15-20 times higher than that of clinical cases (Taylor 1949 and 1952; Rankin1954; and Smith 1954; summarised by Withers 1959). Thus many animals under 30 months of age may be persistently infected with *M. a. paratuberculosis*. There is also thought to be a generalised component to the disease where the bacterium may be found in any part of

the body, and this hypothesis is supported by the findings in this study.

The application of current meat inspection legislation would not specifically identify cases of Johne's disease on the slaughter line. The present study has shown the variability of gross changes seen in Johne's disease at post-mortem examination. The most consistent changes were dilated lymphatics in the gut serosa, intestinal mural thickening and increased thickness and "cobblestoning" of the mucosa. Meat hygiene inspectors have no legal requirement to palpate the intestines and incising the intestines on the slaughter line would be impractical, unhygienic and would lead to increased risk of faecal contamination of the carcass. However the diagnosis of Johne's disease may be facilitated if the mucosa of the terminal small intestine were to be inspected in the gut room where the problems of contamination would be much less. Difficulties could arise as one meat hygiene inspector would be confined to the gut room and there would be a greater chance of contamination of that inspector compared to working on the slaughterline. Therefore the gut inspector on that station would have to change overalls before moving to a station on the slaughterline. Incising into the intestinal lumen would also further increase the potential hazards of workers becoming infected with other enteric pathogens such as *Salmonella sp.* and *Campylobacter sp.* Another problem would be that many abattoirs currently have very small gut rooms where space is limited for line workers. In reality it is often the gut inspection station which is first to sacrifice a man in times of staffing crisis with the inspector of the heads taking responsibility for gut inspection. The result is often that the intestine receives nothing more than a cursory glance.

Correlating gut to carcass may be problematic in situations where the slaughter

line throughput is very high and the time available to dress carcasses and process offal is limited or in any situation which allows a build up of offal in the gut room.

Johne's disease itself does not classify an animal as unfit for human consumption, only the signs of oedema, anaemia, emaciation and poor carcass quality would condemn the carcass. The standard text guide (Gracey et al. 1992, 1999) advises that Johne's disease carcasses may pass for human consumption if the carcass sets but with condemnation of the affected intestines. This study examined a worst case scenario by selecting animals with advanced clinical Johne's disease (Chapter 3). It was found that if judged subjectively, all of the carcasses would pass the current criteria in that they all showed adequate qualities of setting, even an animal (138679) that exhibited marked submandibular oedema in life.

The findings at gross post mortem in this study reflect the diagnostic problems experienced in life. With the exception of "cobblestoning" of the ileum, the pathological changes can be unpredictable and often do not mirror the severity of the clinical disease. Conversely, the opposite situation may occur where the pathological changes and or the burden of mycobacteria within the intestine can be much heavier than would be expected from the signs at post-mortem (Jubb et al. 1993).

Following the above, it is reasonable to hypothesise that Johne's affected carcasses may quite easily make their way into the human food chain. The fact that the carcasses would be classed as poor would mean they would likely be used for manufacturing processes, i.e. they would be used for meat products. This raises concerns as the organism *M. avium-intracellulare* a close relative of *M. a. paratuberculosis,* has been shown to have a higher heat resistance than *M. bovis*, the

organism that pasteurisation techniques are designed to destroy (Merkal et al. 1979). This raises the question as to whether or not the pasteurisation protocol for precooked meat products will adequately destroy the organism given that the products are only rapidly heated and rapidly cooled so that the centre of the products may not reach adequate temperature required to destroy the organism (Merkal et al.1979).

The finding that the prescapular lymph node of one clinically infected animal (136334) was positive for IS900 on PCR supports the concept that Johne's disease does indeed have a generalised component (Alexejeff-Goloff 1923; Matthews 1930; Pearson and McClelland 1955; Doyle 1954 and1958; Lawrence 1956; Kopecky et al 1967; Larsen et al. 1981; Hines et al 1987; Sweeney et al. 1992; Koenig et al. 1993) and thus the organism has the potential to multiply anywhere in the body. With this finding, it is possible to suggest that the organism could be present within major joints of meat and, also with the practice of eating steaks cooked rare, the organism may remain viable within pieces of meat. Even with thorough cooking procedures, the hazard of contamination of hands, utensils and the environment of not only the meat processing plant but that of the consumer remains a possibility.

The inconsistencies with and between the tests used in this study are a common problem for the confirmation of Johne's disease. The difficulty in growing the bacteria in culture, the length of time for adequate growth and the expense of Mycobactin excluded this diagnostic technique from our study even though it is the most consistent method of diagnosis. The BACTEC radiometric culture technique has been shown to be the most sensitive method for diagnosis requiring only 10 organisms/gram of tissue (Sockett et al. 1992) and would therefore be important in future studies because of this aspect and also

because the viability of the organisms could be confirmed. The same study found that PCR method was the poorest method of detection in all the methods tested and needed 10^2 organisms/gram before detection was possible. The reasoning behind the lack of sensitivity could be put down to the extraction technique where the samples may lose organisms with each washing step or the presence of PCR inhibitors within the sample. However the most likely explanation of the poorer sensitivity is the lack of adequate extraction of mycobacterial DNA. It has also been suggested that spheroplastic mycobacteria may remain in the aqueous phase during xylene extraction, which would be an important loss especially where the numbers of organisms being harvested is likely to be small. A report found that if, as well as using xylene extraction, the bacteria were then subjected to lysing by shaking with zirconium beads in a mini bead-beater and guanidine isothiocyanate then used to obtain the DNA (Challans et al. 1994), sensitivity levels of 35-45 mycobacteria/g of tissue detectable by gel electrophoresis could be achieved and that the levels of sensitivity could be increased when using hybridisation methods with ^{32}[P]deoxycytidine triphosphate and then autoradiographed. This protocol could detect 5-10 mycobacteria/g tissue. This level of sensitivity may be needed when examining remote sites of the carcass where numbers of the organism would be expected to be low. This method was not used in the current studies as the apparatus required was not available but nevertheless highlights the possibility for greater detection rates from carcasses studied.

Further Studies

The results from the project have highlighted issues as to the efficiency of sample taking and the diagnostic methods used. Several points that should be addressed in future

studies are:

1. In the future, serial sections should be taken from the samples to eliminate the possibility of missing foci of granulomas within lymph nodes and organs in the body.

2. Cytology (impression smears) should be included again as well as histopathology sections with H and E and ZN-stained sections to confirm whether cytology is a more sensitive method for detecting bacilli within tissues.

3. In this study we used a standard protocol suggested by Grant et al. (1996), for PCR preparation, but sample processing could be modified to optimise the amount of organisms harvested from the samples and obtaining maximal DNA from the organisms by using a ribolyser to disrupt the cell walls and guanidine isothiocyanate to extract the DNA as described by Challans et al. (1994).

4. The PCR product in the future should be digested with restriction enzymes to confirm the restriction pattern is that of *M. a. paratuberculosis* alone.

5. Confirmation of the results would be done by culture of the organism using radiomimetric methods as mycobacterial growth can be detected in less than seven weeks. This would confirm the viability of the organisms within the carcass.

6. As well as subjective meat inspection, carcasses could be weighed to investigate the difference in weight before and after setting. These animals which may be oedematous may show a greater amount of weight loss once "set". Monitoring pH of the carcass as rigor mortis occurs could also be investigated to see if these affected carcasses go through the normal range of pH changes.

7. More animals should be sampled at the OTMS abattoir as five animals is not representative of the population but the exercise showed that such a study within the

abattoir is feasible and could be done on a larger scale.

Conclusion

This study has successfully highlighted the possibility that *M. a. paratuberculosis* could enter the human food chain via infected carcasses that still pass current meat inspection criteria. It has shown that the infection is generalised and that the organism may settle in lymph nodes of the carcass other than those of the intestine. The diagnostic tests used showed inconsistencies between each other but the findings merit further study of this topic using more refined techniques for DNA extraction and sensitive apparatus such as BACTEC radiometric culture for isolating and confirming the presence and viability of mycobacteria within the tissues. Whilst the complete aetiology of Crohn's disease remains to be determined, *M. a. paratuberculosis* remains as a possible factor. The finding of *M. a. paratuberculosis* enteritis in stump tail macaques demonstrating that the organism can cause disease in primates (McClure, Chiodini, Anderson, Swenson, Thayer and Coutu 1987) and the findings in a recent study where 86% of Crohn's disease patients were IS900 positive (Schwartz et al. 2000), underlines this possibility. The veterinary profession should therefore be seen to be making concerted efforts to further their knowledge of Johne's disease and to improve diagnostic methods to help eradicate it from the national herd or, at the very least, from animals slaughtered and processed within abattoirs.

APPENDICES

Appendix 1

Classification of Hide Contamination

Carcasses were graded at post mortem for hide contamination using guidelines published by the Meat Hygiene Service - The Clean Livestock Policy (MHS Operations Manual: Chapter 4 Annex 12)

Cateogory One	Dry coat Clean, no faeces or dirt	Process as normal
Category Two	Dry coat. Light contamination of faeces/dirt. "Hotspots" clean	Process as normal.
Category Three	Significant contamination. Adherent bedding.	Reject for slaughter.
Category Four	Dry, damp coat. Heavily clegged*, significant adherent bedding.	Reject for slaughter. See alternatives. Health mark only when acceptable.
Category Five	Very wet coat. Heavy contamination of dirt and faeces, Heavily clegged*. Lots of adherent bedding.	Reject for slaughter. Reject for slaughter. Return to producer or lair overnight.

"Clegged" means faeces firmly adherent to the animal's coat.

Notes and alternatives.

A coat that is wet but clean is not necessarily hygienically unacceptable for slaughter.

In all cases, contamination of the animal with dirt, faeces or bedding is significant in the following areas called "Hotspots".

Brisket

Abdomen - underside.

Flank.

Ribcage - lower areas and underside.

Hind legs - posterior surface of the hock.

Forelegs- anterior surface of the knee

Neck

Rectal area.

In the circumstances where animals are rejected for slaughter the following action can be taken.

Decrease line speed.

Decrease the number of carcasses on the line at any one time, eg: by leaving alternating spaces between the carcasses.

Pay particular attention to dressing stations at which contamination of the carcass is likely to occur.

The Meat Hygiene Inspector should detain contaminated carcasses.

Appendix 2

The Ziehl-Neelsen Technique

The Ziehl-Neelsen Technique is used for detection of acid-fast bacilli in tissue sections (Drury, Wallington and Cameron 1967).

Preparation.

1. Carbol fuschin:

Basic fuschin	1g
Absolute alcohol	10ml.
5% phenol in distilled water	100ml.

Dissolve the basic fuschin in the alcohol, then mix with the phenol solution. Filter.

Acid-alcohol:

1 per cent. Hydrochloric acid in70 percent. Alcohol.

Counterstain:

Methylene Blue	1g
Glacial acetic acid	1ml.
Absolute ethyl alcohol	20ml.
Distilled water	80ml.

Technique

a. Take sections to water.

b. Flood slide with carbol fuschin, after placing a rectangle of filter paper over the section to prevent precipitation of the stain.Warm the slide until the stain begins to steam. Leave for 5 minutes.

c. Rinse in tap water.

d. Decolourise with acid-alcohol until pale pink and no more colour comes away. A further decolourisation is recommended in neutral 70% alcohol for exclusion of non-pathogenic acid-fast bacilli.

e. Rinse in water.

f. Counter stain with acidified methylene blue with absolute alcohol.

g. Clear in xylene and mount in a synthetic resin medium.

Appendix 3

Gill's Haematoxylin and Eosin Technique

Gill's Haematoxylin and Eosin technique (Drury et al. 1967) is a standard primary staining method for histopathology examination.

Preparation

Distilled water	345ml.
Ethylene Glycol	125ml.
Haematoxylin	3g
Sodium Iodate	0.3g
Aluminium sulphate	26.4g
Glacial Acetic Acid	30ml.

Add in order and mix for one hour. Filter.

Eosin.

Eosin	25g
Saturated aqueous picric acid	250ml
Distilled water	2000ml.
Potassium dochromate	12.5g
Absolute alcohol	250ml.

Add in order.

Technique

1. De-wax sections.
2. Stain in haematoxylin in a jar for 2-20 minutes (depending on the strength of stain).
3. Wash in running water. The section may be examined at this point to to confirm degree of staining.
4. Remove excess stain by decolourising with 0.5-1.0% hydrochloric acid in 70% alcohol for a few seconds.
5. Regain the blue colouring and stop decolourisation by washing in alkaline running tap water for 5 minutes.
6. Stain in 1% aqueous eosin for 1-3 minutes
7. Wash off surplus stain in water
8. Dehydrate in alcohol and clear in xylene.
9. Mount in synthetic resin medium.

ABBREVIATIONS

AGID	Agar Gel Immunodiffusion
CFT	Complement Fixation Test
ELISA	Enzyme-Linked Immunosorbent Assay
H and E	Haemotoxylin and Eosin
IBD	Inflammatory Bowel Disease
OTMS	Over Thirty Month Scheme
PCR	Polymerase Chain Reaction
SBM	Specified Bovine Material
UC	Ulcerative Colitis

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