# *Mycobacterium paratuberculosis* infection in sheep: aspects of diagnosis and immunity

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> ...the essential part of scientific research is dedicated to struggling, not against the evils of Nature, but against the evils begotten by our so-called progress...
>
> *Henri-Frederic Blanc*

# Abstract

Paratuberculosis is a chronic, wasting disease of ruminants caused by *Mycobacterium paratuberculosis*. Programmes aimed at controlling paratuberculosis are based on either vaccination or detection and culling of infected animals. Because of its chronic nature, and the lack of suitable tests for early diagnosis, control of the disease using the latter approach is difficult.

Standard procedures for the isolation of *M. paratuberculosis* are time-consuming and some strains are difficult or impossible to grow. Using the published sequence data of IS*900*, an insertion sequence considered unique for *M paratuberculosis*, a polymerase chain reaction (PCR) assay was developed. With purified extracts of bacterial DNA the PCR assay was found to be highly sensitive and specific. Among 30 bacterial species tested, the assay showed cross-reactivity only with DNA from *M. scrofulaceum*. The possibility of *M. scrofulaceum* causing false positive results in clinical samples from sheep was considered remote, and the assay was subsequently applied to clinical samples. In a study involving 20 sheep suspected of having clinical paratuberculosis, *M. paratuberculosis* DNA was detected in 90% of liver samples and 66% of blood samples from sheep with advanced clinical paratuberculosis. However, in a longitudinal study involving 14 sheep infected experimentally with *M. paratuberculosis*, the PCR failed to consistently detect the target DNA in liver biopsy specimens and blood samples of subclinically infected and clinically affected sheep with mild or moderate extraintestinal infection. Furthermore, the sensitivity of the PCR on samples of ileum and ileocaecal lymph node was similar to that achieved by histological examination.

An experimental model of ovine paratuberculosis, which was developed primarily to validate the PCR assay, created an opportunity to evaluate the diagnostic performance of three commercially available antibody assays for paratuberculosis: complement fixation test (CFT), agar gel immunodiffusion test (AGID), and enzyme-linked immunosorbent assay (ELISA). Two experimental trials demonstrated a limited value of serology for the control of ovine paratuberculosis, as none of the antibody assays was able to detect all sheep with histologically confirmed paratuberculosis. In comparison, the whole-blood interferon-γ (IFN-γ) assay, which

was assessed only during the second trial, detected significantly more experimentally infected sheep and over shorter period of time than any of the serological tests. Furthermore, in a pilot study involving 19 sheep infected experimentally with *M. paratuberculosis*, 18 of the 19 sheep gave positive reactions in the IFN-γ assay on samples of prescapular lymph node (PLN). The PLN-based IFN-γ assay detected significantly more experimentally infected sheep than the CFT, AGID, ELISA or the blood-based IFN-γ assay. Since the specificities of the blood- and PLN-based IFN-γ assay were similar to that of the serological tests, these data indicate the potential utility of this assay, using blood or samples of peripheral lymph nodes, for the detection of sheep exposed to *M. paratuberculosis*. Interestingly, among the 18 sheep tested positive by PLN-based IFN-γ assay, 13 had no histological evidence of paratuberculosis at the time of collection of the PLN samples. In addition, results obtained in a study involving 14 sheep infected experimentally with *M. paratuberculosis* suggest a positive relationship between the magnitude of antigen-induced IFN-γ response in blood and animal's ability to control the infection. Thus, attempts to use this assay in control programmes that are based on testing and culling of positive reactors could result in the removal of animals that have successfully mounted an immune response to the infection.

Vaccination provides an alternative method to test-and-cull programmes of controlling paratuberculosis. Results of a study involving 28 lambs infected experimentally with *M. paratuberculosis*, 14 of which were vaccinated against paratuberculosis with a live-attenuated vaccine 2 weeks postinfection, indicate that vaccination of lambs already exposed to the organism triggered early and strong humoral and cell-mediated immune responses and led to a reduced mycobacterial burden.

But remember,
nothing is actually happening,
and nothing will occur
till the end.

*M. Zablocki*

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At the end, I wish to acknowledge the involuntary contribution made by animals used in the experiments.

**This thesis is dedicated to my parents.**

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# Introduction

## 1.1. Literature review

Paratuberculosis, or Johne's disease, is a chronic, wasting disease of domestic and wild ruminants caused by infection with *Mycobacterium paratuberculosis*, formerly known as *Mycobacterium johnei* (Bang, 1906; Brotherston *et al.,* 1961a; Vance, 1961; Goudswaard, 1971; Boever, 1976; Williams *et al.,* 1983a; Williams *et al.,* 1983b; Gilmour and Nyange, 1989; Belknap *et al.,* 1994; Ridge *et al.,* 1995). The disease is found throughout the world and in some countries is considered economically important. The causative agent of paratuberculosis has also been isolated from cases of granulomatous enteritis in wild rabbits (Greig *et al.,* 1997). It has been suggested that the causal organism may be involved in the aetiology of Crohn's disease in people (Chiodini *et al.,* 1984a; McFadden and Fidler, 1996). Successful experimental infections have been reported in pigs (Larsen *et al.,* 1971), horses (Larsen *et al.,* 1972a), laboratory animals ((Larsen; *et al.,* 1975a; Mokresh *et al.,* 1989; Mokresh and Butler, 1990), lemmings (Larsen and Miller, 1979) and birds (Larsen *et al.,* 1972b; Valente *et al.,* 1997).

### 1.1.1. The organism

The aetiologic agent of paratuberculosis is a small (0.5 to 1.5 μm), slow growing, Gram-positive, acid-fast bacillus (Chiodini *et al.,* 1984b). The organism shares considerable DNA homology with *Mycobacterium avium* and it has recently been proposed by Thorel *et al.* (1990) that the agent should be classified as *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*).

The identification process through cultivation relies on the slow growth rate and the mycobactin-dependence of *M. paratuberculosis* (Chiodini *et al.,* 1984b). This process can be confounded by infections caused by mycobactin-dependent *Mycobacterium avium* strains (Mathews *et al.,* 1977; Barclay and Ratledge, 1983), the great difficulty in

the isolation of some strains of *M. paratuberculosis* (Gunnarsson, 1979; Brooks *et al.*, 1988; Carrigan and Seaman, 1990; Shulaw *et al.*, 1993; Collins *et al.*, 1993a) and variations in mycobactin-dependence of the organism under different culture conditions (Aduriz *et al.*, 1995). It has been suggested that extracellular exochelins (Barclay and Ratledge, 1983) and iron-reductase (Homuth *et al.*, 1998) represent the means of iron acquisition that allow *M. paratuberculosis* to grow *in vivo* in the absence of an external supply of mycobactin.

Recent advances in molecular biology, particularly the identification of IS*900*, an atypical sequence of DNA considered unique to *M. paratuberculosis* (Green *et al.*, 1989), has facilitated the development of DNA probes for rapid and accurate identification of the organism. IS*900* is a 1.45-kb insertion sequence, 15-20 copies of which are present in the *M. paratuberculosis* genome. It lacks terminal inverted repeats, has oriented insertion at homologous target site sequences in the genome and does not induce duplication of the insertion sequence at the target site (Green *et al.*, 1989). IS*900* is implicated in the pathogenicity of *M. paratuberculosis* (Doran *et al.*, 1994) and analysis of its insertion sites suggests that this element targets translation initiation signals to facilitate expression of its HED gene (Doran *et al.*, 1997). The protein product of IS*900*, p43, has recently been purified (Tizard *et al.*, 1992).

The identification of species-specific, immunodominant components of *M. paratuberculosis* may provide the means to improve the diagnostic parameters of serodiagnostic assays for paratuberculosis. Only a relatively small number of antigens, including the bacteroferrin (Brooks *et al.*, 1991), p35 antigen (El-Zaatari *et al.*, 1997a), antigen A (Sugden *et al.*, 1991), LAM (Sugden *et al.*, 1987), 34-kDa antigen (De Kesel *et al.*, 1992; Gilot *et al.*, 1993), hsp 60k (Colston *et al.*, 1994) and hsp 65k (El-Zaatari *et al.*, 1994; El-Zaatari *et al.*, 1995) proteins, have been fully characterised for the organism. One of these, the 34-kDa antigen, has been reported to contain a peptide bearing a species-specific B-cell epitope of *M. paratuberculosis* (De Kesel *et al.*, 1993). Research into species-specific T-cell antigens, along with identification of the role of individual antigens in the pathogenicity of *M. paratuberculosis*, is required to facilitate the development of new generation vaccines and to improve diagnostic tests that measure the cell-mediated immune response.

The difficulty in the isolation of many "ovine strains" (Gunnarsson, 1979; Brooks *et al.*, 1988; Carrigan and Seaman, 1990; Shulaw *et al.*, 1993; Collins *et al.*, 1993a) and the isolation of pigmented colonies primarily from sheep (reviewed by Chiodini *et al.*, 1984b), illustrate phenotypic polarity within the species and suggest genetic divergence. Studies of restriction fragment length polymorphism analysis (RFLP) of *M. paratuberculosis* DNA from limited numbers of isolates have identified genetic heterogeneity between cattle and sheep strains and revealed 3 groups of hybridisation patterns (Collins *et al.*, 1990a; de Lisle *et al.*, 1992; de Lisle *et al.*, 1993). One group includes strains, primarily isolated from cattle, which can be readily cultured. A second group consists of strains from sheep and goats, which are extremely difficult to culture. A third group, identified in sheep, has a hybridisation pattern and culture characteristics intermediate between the first and second group. Other workers (Whipple *et al.*, 1990; Pavlik *et al.*, 1995) could not observe an association between strain polymorphism and the animal species of origin. However, the existence of two major groups of hybridisation patterns, one for strains isolated from cattle and goats and one for "sheep strains" has recently been supported by Thoresen and Olsaker (1994) and Bauerfeind *et al.* (1996). Of critical importance is the question of whether paratuberculosis can be transmitted from cattle to other ruminants and vice versa. Natural transmission of infection from cattle to sheep and goats has been reported (Ris *et al.*, 1987; Ris *et al.*, 1988a). A pigmented "ovine strain" has been isolated from cattle sharing paddocks with sheep (O'Brien *et al.*, 1972). Sheep experimentally infected with bovine isolates have developed paratuberculosis (Kluge *et al.*, 1968; Merkal *et al.*, 1968a) and successful experimental infection of cattle with strains isolated from sheep has also been reported (Taylor, 1953). Presumably, most strains can infect across ruminant species lines (Sweeney, 1996), although some variation of strain pathogenicity in different host species may occur (Saxegaard, 1990).

### 1.1.2. Transmission

Ingestion of *M. paratuberculosis* is believed to be the primary route of infection. Experimental oral infections have produced the disease in cattle, sheep and goats (Nisbet *et al.*, 1962; Payne and Rankin, 1961a; Gilmour *et al.*, 1965; Kluge *et al.*, 1968; Goudswaard, 1971; Allen *et al.*, 1974a; Gilmour *et al.*, 1977). Clinically diseased animals may shed billions of bacilli per day in faeces (Chiodini *et al.*, 1984b). It is

assumed that most animals become infected by ingesting the bacilli from a manure-contaminated udder shortly after birth (Chiodini *et al.,* 1984b; Gay and Sherman, 1992). It has been shown that an oral dose of 100 colony forming units, repeated once a week over 10 weeks, caused intestinal infection in lambs (Brotherston *et al.,* 1961a).

In addition, bacteraemia and dissemination of *M. paratuberculosis* to other organs (Goudswaard, 1971; Hines *et al.,* 1987; Rohde and Shulaw, 1990; Sweeney *et al.,* 1992a; Koenig *et al.,* 1993), including the udder and sex organs, may lead to vertical transmission of infection directly from a dam to offspring. The organism has been isolated from colostrum of 22.2% of asymptomatically infected cows (Streeter *et al.,* 1995) and from milk of 34.6% of cows with clinical paratuberculosis (Taylor *et al.,* 1981). Thus, both milk and colostrum may be a significant source of infection, despite the low concentration of *M. paratuberculosis* organisms that is likely to be present in milk (Sweeney *et al.,* 1992a).

Intrauterine infection of the foetus has been reported in goats (Goudswaard, 1971) and cattle (Seitz *et al.,* 1989; Sweeney *et al.,* 1992b). The percentage of congenitally infected foetuses from cows with clinical paratuberculosis ranged from 26.4% to 63.9% (reviewed by Gay and Sherman, 1992), whereas the infection was detected in only 8.6% of foetuses from asymptomatically infected cows (Sweeney *et al.,* 1992b). Although *M. paratuberculosis* has been recovered from bovine semen (Larsen and Kopecky, 1970), the mechanism of intrauterine transmission is not well understood. An experimental inoculation of the organisms into the uterus after implantation of the zygote has resulted in colonisation of the organ but the organism has not been cultivated from the foetus in such experiments (Merkal *et al.,* 1982).

Experimentally, *M. paratuberculosis* infection has also been transmitted parenterally (by intratracheal, intravenous and/or subcutaneous inoculation) in sheep (Kluge *et al.,* 1968) and cattle (Johnson *et al.,* 1977), resulting in infection in the intestines and elsewhere. Following the injection of *M. paratuberculosis* into the mammary gland the organism was transported to the supramammary lymph nodes in 5 of 6 cows and to the intestine of one cow (Larsen and Miller, 1978a).

### 1.1.3. Clinical manifestation

The incubation period of paratuberculosis is not well defined, however, most clinical cases occur in animals between 3 and 5 years of age (Chiodini *et al.,* 1984b; Gezon *et al.,* 1988; Carrigan and Seaman, 1990). Animals in the early stage of infection show no overt signs of the disease. Since these animals are thought to be abnormally prone to other diseases, they are likely to be culled for reasons unrelated to paratuberculosis and the prevalence of the infection on a farm may be underestimated (Whitlock and Buergelt, 1996).

After a prolonged incubation period, infected animals enter the clinical stage of infection. Initially, in all ruminant species, the disease is characterised by gradual weight loss and progressive weakness that advance to lethargy and emaciation (Vance, 1961; Temple *et al.,* 1979; Morin, 1982; Thomas, 1983; Williams *et al.,* 1983a; Gezon *et al.,* 1988; Gilmour and Nyange, 1989; Whitlock, 1992; Belknap *et al.,* 1994; Ridge *et al.,* 1995; Whitlock and Buergelt, 1996). Some animals may develop submandibular oedema due to hypoproteinaemia (Rajya and Singh, 1961). Unlike cattle, where chronic diarrhoea is a typical manifestation of paratuberculosis, only a small percentage of clinically affected sheep, goats and deer show diarrhoea, and this is usually confined to the terminal stage of the disease (Vance, 1961; Morin, 1982; Gezon *et al.,* 1988; Gilmour and Nyange, 1989; Carrigan and Seaman, 1990). Similar observations have been reported in llamas and alpacas (Belknap *et al.,* 1994; Ridge *et al.,* 1995). The appetite is usually normal, even in animals in an advanced stage of the disease (Whitlock and Buergelt, 1996). Wool break has been reported in sheep (Cranwell, 1993) and deer may show patchy alopecia (Gilmour and Nyange, 1989), presumably due to stress. It has been suggested that for every clinical case on a farm, there are approximately 25 further cows infected (Whitlock, 1992).

### 1.1.4. Pathology

***Gross lesions***

In cattle, small ruminants and deer the gross pathological findings are similar. Clinically affected animals are usually emaciated, have serous atrophy of fat, effusion in the body cavities and, in some cases, submandibular oedema (Stamp and Watt, 1954; Rajya and

Singh, 1961; Vance, 1961; Fodstad and Gunnarsson, 1979; Morin, 1982; Thomas, 1983; Williams *et al.,* 1983b; Gilmour and Nyange, 1989; Carrigan and Seaman, 1990; Clarke and Little, 1996).

The primary macroscopic lesions of paratuberculosis in ruminants are usually confined to the ileum, caecum, colon and draining lymph nodes. The enteric lesions are most common in the terminal ileum and vary from mild, velvety thickening of the mucosa to severe thickening of the bowel with transverse corrugation of the mucosal surface (Rajya and Singh, 1961; Buergelt *et al.,* 1978a; Fodstad and Gunnarsson, 1979; Williams *et al.,* 1983b; Carrigan and Seaman, 1990; Clarke and Little, 1996). In addition, mucosal hyperaemia, erosions and petechiation were observed in deer (Vance, 1961; Gilmour and Nyange, 1989). The mesenteric and ileocaecal lymph nodes are usually enlarged, oedematous and may have focal or diffuse pallor in the cortex (Rajya and Singh, 1961; Buergelt *et al.,* 1978a; Fodstad and Gunnarsson, 1979; Carrigan and Seaman, 1990; Clarke and Little, 1996). The serosal lymphatics are often thickened (Buergelt *et al.,* 1978a; Fodstad and Gunnarsson, 1979; Carrigan and Seaman, 1990 Clarke and Little, 1996) and, in sheep and deer, may have small, white milliary nodules along their length (Gilmour and Nyange, 1989; Carrigan and Seaman, 1990).

***Histopathology***

Histological lesions of paratuberculosis are characterised by the presence of aggregations of large macrophages with abundant granular cytoplasm, often referred to as epithelioid cells, in the intestinal mucosa and submucosa, and in the cortex of mesenteric lymph nodes (Stamp and Watt, 1954; Rajya and Singh, 1961; Vance, 1961; Buergelt *et al.,* 1978a; Fodstad and Gunnarsson, 1979; Morin, 1982; Thomas, 1983; Williams *et al.,* 1983b; Gilmour and Nyange, 1989; Carrigan and Seaman, 1990; Clarke and Little, 1996; Perez *et al.,* 1996). In the intestines, these aggregations of macrophages are accompanied by focal or diffuse infiltration composed of lymphocytes, eosinophils (Rajya and Singh, 1961; Morin, 1982; Thomas, 1983; Clarke and Little, 1996) and occasional neutrophils (Carrigan and Seaman, 1990). Multinucleate giant cells are seen in the intestinal mucosa of cattle (Buergelt *et al.,* 1978a) and sheep (Stamp and Watt, 1954; Rajya and Singh, 1961). In some cases, there are focal aggregates of macrophages and scanty acid-fast organisms (AFO's) in the lamina propria. This type of granulomatous inflammatory reaction is frequently

classified as "paucibacillary, tuberculoid", whereas the diffuse infiltration of the intestinal mucosa and submucosa with macrophages is referred to as "multibacillary, lepromatous", since AFO's are usually numerous in such lesions (Clarke, 1997). The diffuse infiltration of intestinal mucosa is associated with atrophy of villi and a decrease in the number of crypts (Carrigan and Seaman, 1990). In cattle, aggregates of globule leukocytes have been observed in, or around, myenteric ganglion cells (Buergelt *et al.,* 1978a). Extensive fibrosis and necrosis in the mesenteric lymph nodes have been reported to be a feature of paratuberculosis in deer (Gilmour and Nyange, 1989).

In some studies, focal areas of caseation and calcification have been observed in the bowel and mesenteric lymph nodes of sheep and goats (Stamp and Watt, 1954; Rajya and Singh, 1961; Kluge *et al.,* 1968; Fodstad and Gunnarsson, 1979; Morin, 1982; Thomas, 1983; Juste *et al.,* 1994), but other workers have either failed to identify such lesions, or have attributed them to parasitic infestation (Carrigan and Seaman, 1990; Clarke and Little, 1996; Perez *et al.,* 1996).

Gross lesions in the liver have rarely been reported in sheep (Clarke and Little, 1996). However, microgranulomas may be scattered throughout the hepatic parenchyma and there may be mononuclear cell infiltration of the periportal areas in cattle and sheep (Buergelt *et al.,* 1978a; Carrigan and Seaman, 1990; Clarke and Little, 1996). Arteriosclerotic lesions with calcification have sporadically been observed in the aorta and heart in association with paratuberculosis in goats and cattle (Majeed and Goudswaard, 1971; Buergelt *et al.,* 1978a).

In all ruminant species, AFO's are usually numerous within epithelioid cells and multinucleate giant cells in intestinal sections. Fewer AFO's are present in the mesenteric lymph nodes and they are scanty in liver lesions (Buergelt *et al.,* 1978a; Carrigan and Seaman, 1990; Clarke and Little, 1996).

### 1.1.5. Pathogenesis

Although *M. paratuberculosis* does not produce known toxins, the organism is a very successful intracellular parasite, capable of surviving inside macrophages for prolonged

periods (Bendizen *et al.,* 1981; Zurbrick and Czuprynski, 1987). The persistence of *M. paratuberculosis* results in a continuous antigenic stimulation and recruitment of inflammatory cells. There is evidence that this inflammatory infiltration distorts the normal intestinal architecture leading to impairment of nutrient absorption and increased intestinal protein loss (Patterson and Berrett, 1968; Allen *et al.,* 1974a; Allen *et al.,* 1974b). The compensatory increase of protein synthesis in the liver is eventually overwhelmed, resulting in the development of clinical signs (Allen *et al.,* 1974c). However, in some infected animals the severity of clinical signs does not correspond with the magnitude of lesions (Nisbet, 1962; Rankin, 1962). Increased expression of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) mRNA in the blood (Adams and Czuprynski, 1995), and TNF-α, IL-6 and IL-1 mRNA in the intestines of paratuberculous animals (Alzuherri *et al.,* 1996), suggests that cachexia may have an immunological basis.

### 1.1.6. Resistance

It is assumed that most animals which become infected, do so shortly after birth (Chiodini *et al.,* 1984b) and that young animals are most susceptible to infection (Payne and Rankin, 1961b; Larsen *et al.,* 1975b). According to Payne and Rankin (1961b), the ability of adult cattle to eliminate infection is associated with an early development and regression of lesions. After oral inoculation with *M. paratuberculosis* the ileal Peyer's patches serve as portals of entry (Momotani *et al.,* 1988). As these lymphoid organs reach maximum development at birth, and begin to involute from about 3 months of life, the apparent age-related susceptibility to infection has been attributed to the immunomodulating functions of ileal Peyer's patches (Juste *et al.,* 1994). However, this susceptibility is not an all-or-none phenomenon, because adult cattle, either experimentally challenged or exposed to a naturally contaminated environment, can become infected and some even develop the disease (Doyle, 1953; Rankin, 1962).

The magnitude of exposure to *M. paratuberculosis* is the important factor influencing the host's ability to control the infection. Increasing the size of the dose increases both the severity of histological lesions and the occurrence of the clinical disease (Brotherston *et al.,* 1961a). However, sometimes, even animals experimentally inocu-

lated with *M. paratuberculosis* do not develop the disease (Brotherston *et al.,* 1961a; Kluge *et al.,* 1968). The mechanism of this resistance in ruminants is unknown but, in inbred mice, innate resistance to the organism has been shown to be regulated by the *Bcg* gene (Frelier *et al.,* 1990; Veazey *et al.,* 1995). It has been noted that the T-cell profile in the intestines and lymphoid organs of mice susceptible to infection with *M. paratuberculosis* varies significantly from that observed in a resistant strain of mice (Veazey *et al.,* 1996). High activity of acid phosphatase and large phagolysosomes with many degenerate bacteria have been observed in hepatic and splenic macrophages from a resistant strain of mice infected with *M. paratuberculosis*, whereas low acid phosphatase activity and small phagolysosomes with intact mycobacteria have been detected in macrophages from a strain of mice susceptible to infection (Tanaka *et al.,* 1996). A direct relationship between the development of caseous necrotic lesions and clearance of *M. paratuberculosis* has been noted in a resistant strain of mice (Chiodini and Buergelt, 1993). Apart from the *Bcg* gene, numerous single gene defects, including the tumour necrosis factor receptor gene (Pfeffer *et al.,* 1993), the interferon-γ gene (Cooper *et al.,* 1993) and the interferon-γ receptor gene (Kamijo *et al.,* 1993), have been reported to be associated with an increased susceptibility of mice to intracellular pathogens.

Other major influences on the outcome of infection are likely to include the strain virulence, immune status of the host, environmental factors and route of infection. Treatment with glucocorticoids has been reported to be associated with an increased number of *M. paratuberculosis* organisms in the spleen and Peyer's patches of hamsters infected experimentally with the organism (Larsen and Miller, 1978b). It has been noted that infusion with Vitamin D3 exacerbated *M. paratuberculosis* infection, while feeding a low calcium diet reduced the number of viable organisms cultured from the liver and ileum of infected mice (Stabel *et al.,* 1996a). The effect of Vitamin D3 and calcium levels on the susceptibility to infection in natural hosts has not been determined. In another experimental paratuberculosis model in mice, macrophages containing numerous AFO's have been found to have increased amounts of haemosiderin (Lepper *et al.,* 1988), suggesting that multiplication of *M. paratuberculosis* is enhanced in iron-replete as compared with iron-deficient macrophages. However, alteration of the dietary intake of iron had no influence on the development of lesions in cattle (Lepper *et al.,* 1989).

### 1.1.7. Immunology

Generally, the host-parasite interactions that occur during the initial colonisation result in the induction of an immune response, which attempts to eliminate the infection. There is no single response that can effectively deal with all forms of infection and the diversity of effector mechanisms is essential for host survival. It is known that in mice naive CD4 cells can differentiate into T-helper 1 (Th1) or T-helper 2 (Th2) phenotype cells. The Th1 cells secreting interferon-γ (IFN-γ), interleukin-2 (IL-2) and IL-12 are involved in cell-mediated (monocyte/macrophage) response, whereas the Th2 cells producing IL-4 and IL-5 promote antibody production (Bretscher, 1992; Mosmann and Sad, 1996; Elhay and Andersen, 1997; Muraille and Leo, 1998). The classical model of immune response assumes a symmetrical cross-regulation and reciprocal relationship between the Th1 and Th2 phenotypes (Mosmann and Sad, 1996).

***Local immune response in paratuberculosis***

In paratuberculosis, the initial local immune response is believed to be generated by the gut associated lymphoid tissue (Chiodini, 1996). Little is known about immunological events at the site of primary infection. The multifocal character of lesions in early paratuberculosis suggests the existence of multiple infection. The immune system is thought to deal with each focus independently and each focus may generate a distinct immune response at the enteric level (Chiodini, 1996). After infection with *M. paratuberculosis*, a rise of CD8 and gamma/delta T-cells has been observed in tissues of both susceptible and resistant mice. However, higher CD4/CD8 ratios have been noted in a resistant strain of mice (Veazey *et al.,* 1996). Similarly, differences in T-cell profiles at the intestinal level have been observed between sheep with numerous *M. paratuberculosis* organisms and sheep with fewer acid-fast organisms. As compared with normal tissues, lower densities of CD4 and CD8 T-lymphocytes have been detected in intestines from sheep with advanced clinical infection and numerous acid-fast organisms. In contrast, intestines of animals with fewer organisms have been found to have higher CD4/CD8 ratios and increased densities of gamma/delta T-cell subsets (Little *et al.,* 1996). Although the actual role of individual T-cell subsets in immunity to *M. paratuberculosis* in natural hosts is poorly understood, there is convincing evidence that both CD4 and CD8 T-cells are crucial in acquired resistance to *Mycobacterium*

*tuberculosis* and BCG infection in mice (Hubbard *et al.,* 1991; Flynn *et al.,* 1992; Ladel *et al.,* 1995). The contribution of gamma/delta T-cells to immunity against mycobacterial infections is less clear and available data suggest that these lymphocytes have immunoregulatory functions (Chiodini and Davis, 1992; Chiodini and Davis, 1993; D'Souza *et al.,* 1997). It has also been postulated that polymorphonuclear granulocytes participate in the early host response against *M. tuberculosis* (Riedel and Kaufmann, 1997). The antigen-specific response of polymorphonuclear granulocytes in paratuberculosis has not been established.

Antibodies are believed to play little, if any, active role in the resistance to mycobacterial infections (Collins, 1988). In respect to paratuberculosis, research into the mechanism of action of immunoglobulins at the site of infection is limited. An immunohistochemical study has shown increased numbers of IgG and IgM-containing cells but not IgA-containing cells in the ileal lamina propria of paratuberculosis-infected cattle, as compared with control animals (Momotani *et al.,* 1986). There is evidence that antibodies increase the uptake of *M. paratuberculosis* by the M cells of Peyer's patches (Momotani, 1988). Whether this uptake facilitates the establishment of infection or clearance of the organism remains to be determined. It has been shown that cultured bovine monocytes and macrophages require the presence of serum to phagocytose *M. paratuberculosis* (Zurbrick and Czuprynski, 1987). Opsonisation of *M. tuberculosis* with IgG is necessary to trigger phagosome-lysosome fusion for subsequent exposure of ingested bacteria to lysosomal enzymes (Auger and Ross, 1992). Furthermore, an exacerbation of tuberculosis in B cell-deficient mice suggests that these cells contribute to the immunity to mycobacterial infections either through direct cell interaction or through antibody production and neutralisation of mycobacteria-derived immunosupressive factors (Vordermeier *et al.,* 1996).

***Systemic immune response in paratuberculosis***

Most of our understanding of the systemic immune response in paratuberculosis has been derived from studies that compare the diagnostic value of various immunological tests. There is a prolonged delay between oral inoculation with *M. paratuberculosis* and detection of the systemic immune response (Lepper *et al.,* 1989; Juste *et al.,* 1994). The mechanism of this delay is poorly understood, however, it may be due to immuno-

supressive effects of the organism. There is evidence that *M. paratuberculosis* suppresses the delayed hypersensitivity reaction in mice (Kishima *et al.*, 1991). Others have reported a decreased expression of molecules involved in antigen presentation on the surface of intestinal ovine macrophages infected with *M. paratuberculosis* (Alzuherri *et al.* 1997).

The detection of a systemic cell-mediated response precedes detectable antibody production (Lepper *et al.*, 1989). It is believed that the cell-mediated reaction is inversely related to humoral response as the diseases progresses (Chiodini *et al.*, 1984b). Animals which are minimally infected frequently react positively to tests that measure cell-mediated immunity but do not react on serological testing (Buergelt *et al.*, 1977; Buergelt *et al.*, 1978b; de Lisle *et al.*, 1980a; de Lisle *et al.*, 1980b; de Lisle and Duncan, 1981; Billman-Jacobe *et al.*, 1992). In contrast, some seropositive animals have no detectable cellular response (Buergelt *et al.*, 1977). The cellular immune system is crucial for controlling intestinal multiplication of *M. paratuberculosis* in mice (Hamilton *et al.*, 1989; Hamilton *et al.*, 1991)). A negative relationship between levels of intestinal infection and the magnitude of systemic cell-mediated reaction, measured by the skin test, has been noted in sheep and cattle (Gilmour and Brotherston, 1966; Gilmour *et al.*, 1977; Wentnik *et al.*, 1993). In comparison, a positive relationship has been determined between the presence of acid-fast bacilli in intestinal lesions and positivity to serological tests for the detection of antibodies to *M. paratuberculosis* (Clarke *et al.*, 1996; Perez *et al.*, 1997). This seems to support the doubtful contribution of antibodies to the immunity to *M. paratuberculosis.* Some investigators even consider that excessive local production of immunoglobulins in the intestinal mucosa may account for diarrhoea and participate in the pathogenesis of bovine paratuberculosis (Momotani *et al.*, 1986). The humoral response is thought to be stimulated by release of large amounts of antigen following the intracellular death of bacilli (Collins, 1988). Strong IgG and IgM responses but weak IgA reactions were found in the serum of paratuberculosis-infected cattle (Abbas and Riemann, 1988).

The inability to detect cellular responses in animals that are still able to produce specific antibodies is intriguing. Detection of a humoral suppressor of lymphocyte transformation factor in paratuberculous cows (Davies *et al.*, 1974) and reproduction of cutaneous desensitisation after local antigen overloading (Larsen and Johnson, 1949) suggest that

this anergy is a result of the complex host-parasite interactions. In addition, the cell-mediated immune reaction may be affected by environmental factors. In vitamin $B_{12}$-deficient lambs following vaccination against paratuberculosis, higher lymphoblastic responses against *M. paratuberculosis* have been detected in animals supplemented with cobalt than in non-supplemented lambs, while no differences between these 2 groups were found in antibody production (Vellema *et al.,* 1996). An impairment of cellular immunity has been shown in paratuberculosis-infected cows (Wentnik *et al.,* 1988) and mice (Follett and Czuprynski, 1990) treated with glucocorticoids. Relapses of the disease which sometimes occur after parturition in cows are believed to result from stress-related interference with cell-mediated immunity (Chiodini *et al.,* 1984b).

***Role of cytokines in immunity in infection with Mycobacterium paratuberculosis***

Data derived from gene knock-out mouse models, and other studies, convincingly indicate that IFN-γ produced by T lymphocytes plays an essential role in the defence against intracellular pathogens (Cooper *et al.,* 1993; Kamijo *et al.,* 1993; Flesch *et al.,* 1995). The earlier decrease in the number of viable *M. paratuberculosis* bacilli in IFN-γ pre-treated bovine monocytes, as compared with untreated control monocytes (Zhao *et al.,* 1997), suggests that this cytokine contributes to acquired resistance against the organism in natural hosts. Others have reported that IFN-γ neither increased nor restricted intracellular growth of *Mycobacterium paratuberculosis* in bovine macrophages, however, the restriction of bacillary growth has been observed in monocytes incubated with virus-induced crude bovine interferon or recombinant bovine IFN-α (Zurbrick *et al.,* 1988). Although there is evidence that treatment with IFN-γ induces release of free radicals from bovine monocytes infected with *M. paratuberculosis* (Zhao *et al.,* 1997), the mechanism of *in vitro* growth inhibition of the organism in bovine monocytes appears to be independent of phagocyte oxidative activity (Zurbrick *et al.,* 1988; Zhao *et al.,* 1997). Nevertheless, it has been reported by Hines *et al.* (1996) that IFN-γ partly interferes with the inhibition of microbicidal functions of murine macrophages incubated with a glycolipid compound derived from *M. avium* (formerly known as *M. paratuberculosis* strain 18), which is known to scavenge free radicals (Scherer *et al.,* 1997).

The *in vivo* administration of a monoclonal antibody against the interleukin-1 (IL-1)

receptor has been found to inhibit the ability of mice to eliminate *M. paratuberculosis* (Kenefick *et al.,* 1994). Although this suggests participation of IL-1 in the defence against the organism in mice, the contribution of this cytokine to immunity against *M. paratuberculosis* in natural hosts is less clear. An increased production of IL-1 by monocytes from diseased cattle has been reported (Kreeger *et al.,* 1991). Similarly, elevated levels of IL-1β and TNF-α mRNA have also been observed in intestines from sheep having clinical paratuberculosis (Alzuherri *et al.,* 1996). Thus, the role of cytokines in control of intracellular growth of *M. paratuberculosis* appears to be complex. An *in vitro* study has shown that TNF-α can either restrict or encourage growth of *Mycobacterium paratuberculosis*, depending on the concentration and time interval used (Stabel, 1995).

It is worth mentioning that *in vitro* studies investigate certain aspects of the immune response in artificial environment and often in isolation from other immunological events. Such experiments may provide little, if any, insight into complex interactions between various components of the immune system. Thus, interpretation of results derived from *in vitro* studies is frequently difficult and any extrapolations to *in vivo* immunological events must be done with caution.

## 1.1.8. Diagnosis of paratuberculosis

### 1.1.8.1. Methods that identify *Mycobacterium paratuberculosis* and provide direct evidence of infection

***Culture***

The isolation of *M. paratuberculosis* from an animal provides the definitive diagnosis of infection with the organism. Since recovery from infection is believed not to occur, the isolation of the organism is assumed to be 100% specific (Collins, 1996). The estimated sensitivity of conventional faecal culture is approximately 50% in cattle (Sockett *et al.,* 1992). However, culture techniques are not standardised and the diagnostic specificity and proficiency may vary between laboratories, depending on quality control practices and methods used to identify slow growing mycobacterial

isolates (reviewed by Collins, 1996). Contamination rates reported by Kim *et al.* (1989) ranged from 4.1 to 10.8%. The major disadvantage of conventional culture is that the organism requires 8 to 16 weeks, or longer, to grow (Bauerfeind *et al.*, 1996).

An alternative technique for isolating *M. paratuberculosis* is a radiometric culture method, known as the BACTEC system. This method has been reported to provide results within 7 weeks, and is considered to be more sensitive than conventional methods (Collins *et al.*, 1990b; Damato and Collins, 1990; Sockett *et al.*, 1992). However, some strains of the organism, especially strains isolated from sheep, are very difficult to grow (Gunnarsson, 1979; Brooks *et al.*, 1988; Carrigan and Seaman, 1990; Collins *et al.*, 1993a; Shulaw *et al.*, 1993) and may not be detected by either method. In a recent study, the use of Middlebrook 7H11 medium has resulted in the isolation of *M. paratuberculosis* from 78% of clinically affected sheep (Aduriz *et al.*, 1995). This cultivation method may prove to have wider diagnostic application.

***Polymerase Chain Reaction***

Recent advances in molecular biology, in particular the development of polymerase chain reaction (PCR) technology, have offered new hope for the development of rapid, sensitive and specific tests for a range of diseases. In case of paratuberculosis, the identification of IS*900* has precipitated the emergence of PCR assays for detection of the organism in faeces (Vary *et al.*, 1990; Sockett *et al.*, 1992; van der Giessen *et al.*, 1992; Collins *et al.*, 1993b). These tests were reported to be of high specificity but they lacked sensitivity (Sockett *et al.*, 1992; van der Giessen *et al.*, 1992; Collins *et al.*, 1993b). The low sensitivity has been attributed to the low volume of sample tested and inhibition of the PCR by constituents of faeces (Vary *et al.*, 1990; van der Giessen *et al.*, 1992).

The problem of PCR inhibition led to the development of a combined BACTEC radiometric culture-PCR system (BACTEC-PCR) and a hybridisation capture-PCR method (HC-PCR). The BACTEC-PCR combination has been reported to be both more sensitive and more rapid than the routine culture (Cousins *et al.*, 1995; Whittington *et al.*, 1998). The HC-PCR is expected to provide sensitivity advantages over the traditional PCR technique (Millar *et al.*, 1995).

***Microscopic examination***

Direct microscopy of faecal and mucosal smears stained by the Ziehl-Neelsen method provides only a presumptive indication of paratuberculosis and is not reliable for either confirming or excluding the diagnosis (Merkal *et al.,* 1968b; Koh *et al.,* 1988; Ris *et al.,* 1988b).

Histological examination of tissues stained with haematoxylin and eosin, and by the Ziehl-Neelsen method is usually limited to dead animals and has been reported to be less sensitive than tissue culture in goats (Fodstad and Gunnarsson, 1979) and cattle (Koh *et al.,* 1988). Furthermore, positive results require confirmation of infection by isolation of the organism, with the exception of sheep in which histology, in spite of its limitations, is considered to be the "gold standard" diagnostic technique due to the difficulty of growing "ovine strains". Immunohistochemical techniques provide an adjunct to standard histological methods for diagnosis of paratuberculosis (Massone *et al.,* 1990; Navarro *et al.,* 1991; Thoresen *et al.,* 1994; Stabel *et al.,*1996b; Coetsier *et al.,* 1998).

### 1.1.8.2. Tests which provide indirect evidence of infection with *Mycobacterium paratuberculosis*

***Serological tests***

There are three widely used tests for the serodiagnosis of paratuberculosis: complement fixation test (CFT), agar gel immunodiffusion test (AGID), and enzyme-linked immunosorbent assay (ELISA).

Diagnostic performance of a test has two components; sensitivity and specificity. These components are usually estimated against results obtained by histology or culture of the organism. Accurate determination of sensitivity and specificity of serological tests is difficult due to the slow, progressive nature of paratuberculosis and the limitations of culture and histology to accurately detect early infection. This could be partly responsible for the disagreement between published reports of sensitivity and specificity. However, most likely, the wide range of values reflects the application of different criteria to establish the infection status of animals, as illustrated in Tables 1.1, 1.2 and 1.3. The use of different antigens may also contribute to the variation in reported accuracy of serological tests (Molina *et al.,* 1991; Sugden *et al.,* 1991).

**Table 1.1. Published specificities and sensitivities of serological tests for the diagnosis of paratuberculosis in cattle.**

| TEST | Reported sensitivity(%) | Infection status criterion | Reported specificity(%) | Infection-free status criterion | Reference |
|---|---|---|---|---|---|
| CFT | 63.9 | Faecal culture | 87.2 | Faecal culture | Colgrove et al. 1989 |
| AGID | 27.8 | (+ve) animals | 96.8 | (-ve) animals | |
| ELISA | 94.4? | | 82.7 | | |
| AGID | 33 | Faecal culture (+ve) subclinical cases | 97.9 | Faecal culture (-ve) animals | Sherman et al. 1989 |
| AGID* | 42 | Faecal culture | 92 | Faecal culture | Tsai et al. 1989 |
| m-AGID* | 49 | (+ve) animals | 90 | (-ve) animals | |
| ELISA* | 65 | | 86 | | |
| ^ELISA* | 68 | | 84 | | |
| ELISA* | 57 | Faecal culture and/or histologically (+ve) animals | 98.9 | Faecal culture (-ve) animals | Milner et al. 1990 |
| CFT | 10.8 | Faecal and tissue | 97.5 | Animals from herds | Sherman et al. 1990 |
| AGID | 18.9 | culture (+ve) subclinical cases | 99.4 | with no history of paratuberculosis | |
| ELISA* | 65 | Faecal culture (+ve) clinical cases | 99.8 | Animals from a region believed to be free of paratuberculosis | Cox et al. 1991 |
| #ELISA* | 88.2 | Histologically | 99.8 | Animals from a region | Ridge et al. 1991 |
| CFT | 83.4 | confirmed clinical cases | 96.9 | believed to be free of paratuberculosis | |
| #ELISA* | 59.7 | Subclically infected | as above | as above | as above |
| CFT | 57.3 | faecal culture (+ve) animals | | | |
| #ELISA* | 48.8 | Subclinical cases | as above | as above | as above |
| CFT | 21.4 | confirmed by culture and/or histology | | | |
| ELISA* | 67.9 | Subclically infected | 99.9 | Animals from herds | Yokomizo et al. 1991 |
| CFT | 26.3 | faecal culture (+ve) animals | 97.6 | with no history of paratuberculosis | |
| ^ELISA | 65.9 | Faecal culture | 91.4 | Faecal culture | Bech-Nielsen et al. 1992 |
| ^ELISA* | 34.1 | (+ve) animals | 97.8 | (-ve) animals | |
| ELISA | 71 | Faecal culture | 83 | Faecal culture | Spangler et al. 1992a |
| AGID | 38 | (+ve) animals | 100 | (-ve) animals | |
| r-ELISA | 70 | Samples from | 95 | Tuberculous cattle and | Vannuffel et al. 1994 |
| #ELISA* | 77 | paratuberculous cattle | nr | animals from herds | |
| #ELISA* | 80 | obtained from | nr | with no history | |
| #ELISA* | 80 | the U.S. National | nr | of paratuberculosis | |
| #ELISA* | 87 | Repository for | nr | | |
| CFT | 57 | Paratuberculosis | nr | | |
| AGID | 53 | Specimens | nr | | |
| #ELISA* | 45 | Faecal culture (+ve) both subclinical and clinical cases | 99 | Animals (-ve) by histology and faecal and tissue culture | Sweeney et al. 1995 |

? - includes suspicious and positive results, * - preadsorption of sera with *M. phlei* antigen, # - commercially available kit for cattle, ^ - dot-blot ELISA, r - recombinant antigen, m - modified, (+ve) - positive, (-ve) - negative, nr - not reported.

**Table 1.2. Published specificities and sensitivities of serological tests for the diagnosis of paratuberculosis in sheep.**

| TEST | Reported sensitivity (%) | Infection status criterion | Reported specificity (%) | Infection-free status criterion | Antigens tested | Reference |
|---|---|---|---|---|---|---|
| AGID | 81.8 | Animals | 88.1 | Animals | M.ptb antigen D | Sugden at al. 1989 |
| CFT | 22.7 | (+ve) by | 100 | (-ve) by | M.ptb polysaccharide | |
| ELISA | 72.7 | histology | 88.1 | histology | M.ptb LAM | |
| | | and/or culture | | and/or culture | | |
| ELISA | 64 | Animals | 90 | Animals | M.ptb LAM | Sugden et al. 1991b |
| ELISA | 82 | (+ve) by | 90 | (-ve) by | M.ptb antigen A | |
| ELISA | 100 | histology | 90 | histology | M.ptb antigen D | |
| | | and/or culture | | and/or culture | | |
| AGID | 100 | Clinilcal cases | 100 | Specific | M.ptb sonicate | Hilbink et al. 1994 |
| CFT | 100 | with gross | 96 | pathogen-free | M.ptb homogenate | |
| ##ELISA | 100 | lesions and | 98 | sheep | | |
| ##ELISA* | 92.3 | (+ve) | 98 | | | |
| ELISA | 100 | faecal | 91 | | M.ptb sonicate | |
| ELISA* | 100 | smears | 91 | | as above | |
| AGID | 85.7 | Clinilcal cases | 97.5 | Animals from | M.ptb sonicate | as above |
| CFT | 85.7 | confirmed by | 100 | flocks with no | M.ptb homogenate | |
| ##ELISA | 85.7 | histology | 99.5 | history of | | |
| ##ELISA* | 80.9 | | 100 | paratuberculosis | | |
| ELISA | 85.7 | | 99.5 | | M.ptb sonicate | |
| ELISA* | 85.7 | | 99.5 | | as above | |
| #ELISA* | 48 | Animals | 95 | Animals | | Dubash et al. 1995 |
| | | (+ve) by | | (-ve) by | | |
| | | histology | | histology | | |
| | | and/or culture | | and/or culture | | |
| CFT | 48.2 | Faecal culture | 74.5 | Faecal culture | | Benazzi et al. 1996 |
| | | (+ve) animals | | (-ve) animals | | |
| ELISA* | 86.4 | Animals (+ve) | nr | | | Clarke et al. 1996 |
| AGID | 100 | by histology | | | | |
| | | (multibacillary) | | | | |
| ELISA* | 50 | Animals (+ve) | as above | | | as above |
| AGID | 30 | by histology | | | | |
| | | (paucibacillary) | | | | |
| ELISA | 48.4 | Suspected | 88.9 | Suspected | protoplasmic (M.ptb) | Perez et al. 1997 |
| AGID | 37.1 | clinical cases | 100 | clinical cases | as above | |
| | | confirmed by | | without | | |
| | | histology | | histological | | |
| | | | | lesions | | |

* - preadsorption of sera with *M. phlei* antigen, # - commercially available kit for cattle, ## - commercially available kit for cattle modified for sheep, **(+ve)** - positive, **(-ve)** - negative, **nr** - not reported, **M.ptb** - *M.paratuberculosis.*

**Table 1.3. Published specificities and sensitivities of serological tests for the diagnosis of paratuberculosis in goats.**

| TEST | Reported sensitivity (%) | Infection status criterion | Reported specificity (%) | Infection-free status criterion | Antigens tested | Reference |
|---|---|---|---|---|---|---|
| **AGID** | **77.5** | Clinical cases confirmed by histology and/or tissue culture | **80** | Animals from a paratuberculosis - infected herd (-ve) by histology and/or tissue culture | protoplasmic M.ptb/strain 18 | Sherman and Gezon, 1980 |
| **ELISA*** | **87** | Clinical | **100** | Animals (-ve) | sonicate (M.ptb/316) | Milner et al.1989 |
| **AGID** | **73** | cases | **100** | by histology | as above | |
| **CFT** | **80** | confirmed by histology | **36** | | as above | |
| **ELISA*** | **87.5** | Clinical and | **94.4** | Animals (-ve) | sonicate (M.ptb) | Molina et al. 1991 |
| **ELISA** | **87.5** | subclinical cases | **91.7** | by histology and/or | as above | |
| **ELISA*** | **64.7** | confirmed | **93.1** | tissue | protoplasmic (PPA-3) | |
| **ELISA** | **88.2** | by histology and/or tissue culture | **94.4** | culture | as above | |
| **##ELISA*** | **54** | Animals with IS900 DNA detected in faeces | **100** | Animals from presumably uninfected herds | | Burnside and Rowley, 1994 |

* - preadsorption of sera with *M. phlei* antigen, ## - commercially available kit for cattle modified for goats, (-**ve**) - negative, **M.ptb** - *M.paratuberculosis*

In the past, the specificity of serological tests for paratuberculosis has been reported to be low, partly due to the use of crude antigens containing epitopes common to other organisms (reviewed by Chiodini *et al.*, 1984b). Technical modifications to those tests have resulted in some improvement in the specificity of antibody assays. In case of bovine ELISA, the adsorption of sera with *M. phlei* to remove non-specific, cross-reacting antibodies resulted in higher specificity, however, this was associated with some loss of sensitivity (Tsai *et al.*, 1989; Bech-Nielsen *et al.*, 1992). In comparison, the incorporation of the *M. phlei* adsorption step into tests for the diagnosis of paratuberculosis in sheep and goats resulted in little, if any, increase in specificity (Molina *et al.*, 1991; Hilbink *et al.*, 1994).

The most important factor affecting the sensitivity of a test is the concentration of antibodies required to give a positive result. This property varies between tests (Collins,

1996), and therefore, the test sensitivity may differ depending on the stage of the disease and the kinetics of the humoral response. In addition, it is suspected that in sheep each serological test detects a different population of antibodies to *M. paratuberculosis* (Stehman, 1996). The sensitivity of all serological tests for paratuberculosis is low in preclinical cases but improves with progression of the mycobacterial burden (de Lisle *et al.,* 1980b; Sherman *et al.,* 1989; Tsai *et al.,* 1989; Sweeney *et al.,* 1995; Clarke *et al.,* 1996; Perez *et al.,* 1997). For example, sensitivity of ELISA in one study was only 15% in low-level faecal shedders, whereas in clinically affected, heavy faecal shedders the sensitivity was 87% (Sweeney *et al.,* 1995).

As previously noted, comparison of the diagnostic performance of antibody assays is difficult. Nevertheless, data available indicate that the ELISA is the most sensitive and specific test for the serological diagnosis of paratuberculosis in cattle (Colgrove *et al.,* 1989; Tsai *et al.,* 1989; Yokomizo *et al.,* 1991; Spangler *et al.,* 1992a). Among the available antibody assays for the diagnosis of paratuberculosis in small ruminants, the CFT, AGID and ELISA have been reported to have similar discriminatory powers for detecting clinical cases (Milner *et al.,* 1989; Hilbink *et al.,* 1994; Perez *et al.,* 1997). The AGID and ELISA seem to be more sensitive in identifying subclinically infected sheep than the CFT (Sugden *et al.,* 1989; Hilbink *et al.,* 1994). Unlike the CFT and ELISA, the AGID does not seem to detect antibodies to *Corynebacterium pseudotuberculosis* (Sugden *et al.,* 1989; Shulaw *et al.,* 1993; Hilbink *et al.,* 1994).

Attempts to apply the ELISA to milk for diagnosing paratuberculosis have not been encouraging (Sweeney *et al.,* 1994). The use of a recombinant species-specific antigen, a 362 polypeptide, does not appear to provide any sensitivity advantage over commercially available tests using crude or purified antigens for the detection of bovine antibodies to *M. paratuberculosis* (Vannuffel *et al.,* 1994). The utility of a recombinant p35 antigen in the serodiagnosis of animals with paratuberculosis at all stages of infection remains to be determined (El-Zaatari *et al.,* 1997a).

Since evidence of a systemic humoral response in most cases of paratuberculosis reflects the more advanced stages of infection, serological tests are considered useful only in identifying infected herds and animals that pose the greatest risk of spreading the dis-

ease (Hilbink *et al.,* 1994). However, serological tests provide only indirect evidence of infection by detecting antibodies to *M. paratuberculosis* in the serum. Furthermore, the immune response in paratuberculosis is erratic, and even animals that are in the advanced clinical stage are not always identified correctly by antibody assays (Sweeney *et al.,* 1995). These inherent shortcomings limit the use of the antibody assays in predicting infection status in individual animals and restrict their application in control programs (Kreeger, 1991; Hilbink *et al.,* 1994; Dubash *et al.,* 1995).

***Tests that measure cell-mediated immune response***

While antibodies rise as the disease progresses, evidence of cell-mediated immune response (CMI) is usually detectable in the early stage of infection with *M. paratuberculosis* (Lepper *et al.,* 1989). In the past, the degree of delayed-type hypersensitivity and a febrile response after intradermal or intravenous injection of *M. paratuberculosis* antigen were used to measure CMI. The reported sensitivity and specificity estimates of skin testing vary widely (Larsen *et al.,* 1963; Merkal *et al.,* 1968b; de Lisle *et al.,* 1980a). Both the intravenous and intradermal test lack accuracy (Larsen *et al.,* 1963; Larsen and Kopecky, 1965; Merkal *et al.,* 1968b; de Lisle *et al.,* 1980a; Benedictus and Bosma, 1985; Fawcett *et al.,* 1995). These tests may also alter the immune status of the animal tested (Larsen and Johnson, 1949; Merkal *et al.,* 1970). Consequently, attempts have been focused on the development of alternative methods to measure CMI.

The lymphocyte transformation test has been shown to detect lightly infected animals that were frequently seronegative (Buergelt *et al.,* 1977; Buergelt *et al.,* 1978b; de Lisle and Duncan, 1981; Hintz, 1981). However, this test requires the use of radioactive material and, as with the leukocyte migration inhibition test (Bendixen, 1977), has not reached wide acceptance for the diagnosis of paratuberculosis. The recent advent of an *in vitro* assay, which is based on the antigen-induced production of IFN-γ, has provided an alternative method to measure CMI (Wood *et al.,* 1990). Studies on a limited number of cows indicate that the IFN-γ assay may be an important tool for identifying of animals subclinically infected with *M. paratuberculosis* (Billman-Jacobe *et al.,* 1992; Stabel, 1996).

### 1.1.9. Control

Generally, all programmes aimed at controlling infectious diseases attempt to prevent the transmission of infectious agents from infected to healthy animals and paratuberculosis is no exception. Removal or segregation of animals diagnosed as being infected, obtaining infection-free replacements, improved hygiene, and separation of young stock from potential sources of infection, have been recommended as means of managing the disease (Merkal *et al.,* 1975; Thomas, 1983; Collins, 1994; Rossiter and Burhans, 1996). In addition, offspring from infected female animals should be culled, because of a high risk of transmission of infection from such dams (Seitz *et al.,* 1989; Sweeney *et al.,* 1992a).

The stringency of a control programme influences the final outcome. Schemes based on removal of faecal shedders alone have not controlled the shedder-rate in subsequent years (Sherman and Gezon, 1980; Thomas, 1983). Adoption of a strict test-and-cull programme, combined with hygiene and husbandry improvements, has been reported to eliminate paratuberculosis from a herd of goats (Gezon *et al.,* 1988).

### 1.1.10. Vaccination

Vaccination provides an alternative method to test-and-cull programmes of controlling paratuberculosis. The objective of vaccination is to prevent the infection from becoming established by inducing immunity against the organism. The mechanism of action of paratuberculosis vaccines is poorly understood. There is evidence that immunity correlates with the ability of vaccinated animals to quickly develop a strong delayed-type hypersensitivity reaction (Gilmour and Brotherston, 1966). It has also been noted that early development and regression of granulomas is associated with the elimination of infection in vaccinated sheep (Juste *et al.,* 1994).

A number of vaccines containing various preparations of live attenuated *M. paratuberculosis* strains (Gilmour and Brotherston, 1966; Saxegaard and Fodstad, 1985; Hilbink and West, 1990; Cranwell, 1993; Molina *et al.,* 1996), heat-killed *M. paratuberculosis* (Brotherston *et al.,* 1961b; Gilmour and Brotherston, 1966) and heat-killed *M. paratuberculosis* strain 18 (currently known as *M. avium* serovar 2)

organisms (Doyle, 1964; Larsen *et al.,* 1964) have been evaluated. In addition, the immunogenicity of disrupted fragments (Larsen *et al.,* 1969) and fractions of these organisms (Gilmour and Brotherston, 1966) has been tested. In a field trial the whole-cell vaccine provided better protection than the fractionated-cell vaccine (Larsen *et al.,* 1978). The protoplasmic fraction of *M. paratuberculosis*, the residues of alcohol/potassium hydroxide extraction and the oil-extracts of the organism have been found to induce little immunity, while the heat-killed bacterin and the methanol-treated *M. paratuberculosis* organisms have caused high, and comparable, degrees of protection against superimposed infection (Gilmour and Brotherston, 1966).

Vaccination has been used to control paratuberculosis in goats, sheep, cattle and deer and resulted in reduction of the incidence of clinical disease, lower mortality (Sigurdsson, 1960; Doyle, 1964; Wilesmith, 1982; Saxegaard and Fodstad, 1985; Breukink *et al.,* 1990; Fawcett *et al.,* 1995; Cranwell, 1993) and decreased faecal shedding of *M. paratuberculosis* (Körmendy, 1994), but complete elimination of infection has not been achieved. Since most data on vaccination are incomplete and fragmented, little is known about the efficacy of vaccination. The exception is a few studies in which mortality due to paratuberculosis has been reduced by approximately 93% in vaccinated sheep (Sigurdsson, 1960) and by 90% in vaccinated cattle (Schaik *et al.,* 1996). In a field trial using a live vaccine in goats, the infection rate, as determined by post-mortem examinations, was 1% in the vaccinates and 53% in the unvaccinated animals (Saxegaard and Fodstad, 1985). In another field trial using killed bacterins in cattle, 9.8% vaccinates and 56.4% of the control unvaccinated animals were found to be infected at slaughter (Larsen *et al.,* 1978). According to Saxegaard and Fodstad (1985), the use of vaccine alone cannot control paratuberculosis and other procedures such as improved hygiene, appropriate husbandry and removal of faecal shedders and clinically affected animals should be implemented as well. As a result, it is frequently difficult to determine if any beneficial effects are due to the change of management or to the vaccine. Furthermore, it is expected that in endemically infected herds many animals are exposed to the infection before vaccination (Chiodini *et al.,* 1984b). According to Gilmour *et al.* (1965) the use of vaccine in animals already infected with *M. paratuberculosis* does not modify the infection.

Vaccination as a control option for paratuberculosis does have disadvantages. The op-

tion of monitoring infection status with serological tests is eliminated in vaccinated herds or flocks (Hilbink and West, 1990; Spangler *et al.,* 1991). Furthermore, vaccination leads to sensitisation of animals to both avian and bovine tuberculin. Thus, vaccination could interfere with tuberculosis control schemes. However, the reaction to avian tuberculin in animals vaccinated against paratuberculosis is usually equal to, or greater than the reaction to bovine tuberculin (Doyle, 1964; Milestone, 1988).

Vaccination is accomplished by subcutaneous injection of vaccine and an additional disadvantage of the use of vaccine is the development of extensive fibrocaseous lesions at the injection site (Gilmour and Brotherston, 1966; Milestone, 1988). In addition, severe localised reactions after accidental self-inoculation present a human health risk. Attempts to immunise animals using an alternative, oral, route of vaccination have not been encouraging (Brotherston *et al.,* 1961b; Nisbet *et al.,* 1962).

### 1.1.11. Treatment

In spite of the development of many new antimycobacterial agents over the last 50 years and the *in vitro* sensitivity of *M. paratuberculosis* to most of these therapeutic agents (Larsen and Vardman, 1950; Larsen and Vardman, 1952; Larsen and Vardman, 1953; Rankin, 1953; Eidus and Denst, 1964; Gilmour, 1966; Mandell and Sande, 1985), chemotherapy for paratuberculosis has usually been palliative (Larsen and Vardman, 1950; Larsen and Vardman, 1952; Rankin, 1953; Gilmour, 1970; Merkal and Larsen, 1973; Baldwin, 1976; Gezon *et al.,* 1988), and rarely successful in clinically affected animals (Jean, 1996). It is assumed that the discrepancy between the *in vitro* and *in vivo* sensitivities is due to inaccessibility of intracellularly located *M. paratuberculosis* (Cocito *et al.,* 1994). However, if treatment is instituted in the early stage of infection recovery from paratuberculosis may occur (Jean, 1996). Riminiphenazine has inhibited the development of clinical disease in experimentally inoculated sheep (Gilmour, 1966). Nevertheless, because of the cost of therapy and the requirement of daily medication the treatment of paratuberculosis may be recommended only for valuable or companion animals (Cocito *et al.,* 1994; Jean, 1996).

### 1.1.12. Economic impact

An accurate analysis of the economic impact of paratuberculosis is hampered by the prolonged course of infection and predominantly subclinical nature of the disease. Several investigators have reported reduced milk production in clinically and subclinically infected cattle (Buergelt and Duncan, 1978; Benedictus *et al.*, 1987; Hutchinson, 1988; de Lisle and Milestone, 1989; Körmendy *et al.*, 1989; Wilson *et al.*, 1993; Nordlund *et al.*, 1996). According to three studies (Buergelt and Duncan, 1978; Körmendy *et al.*, 1989; Wilson *et al.*, 1993), paratuberculous cattle are culled earlier than their herdmates. This premature culling results in increased costs of obtaining replacements and important, but difficult to quantify, costs associated with loss of potentially valuable genetic material. Some workers have reported impaired reproductive performance and increased mastitis incidence in infected animals (Merkal *et al.*, 1975; Abbas *et al.*, 1983), but others have shown no association between paratuberculosis and either infertility or mastitis (de Lisle and Milestone, 1989; Wilson *et al.*, 1993). Although the economics of paratuberculosis are uncertain, anecdotal evidence, experience, and data from vaccination trials (Schaik *et al.*, 1996) suggest that the impact of the disease on farm profitability can be substantial in heavily infected properties.

### 1.1.13. Crohn's disease in people and *Mycobacterium paratuberculosis*

Despite recent advances in the study of Crohn's disease, the cause of this chronic inflammatory bowel disease (IBD) remains undetermined. Genetic, nutritional, environmental, immunological and microbial influences have been suggested (reviewed by Thompson, 1994; Elson *et al.*, 1995; Koutroubakis *et al.*, 1996). Similarly, the aetiology of human ulcerative colitis, another IBD, is not known. Among the microbial factors, various bacteria (reviewed by Thompson, 1994) and viral agents (Wakefield *et al.*, 1995; Ekbom *et al.*, 1996; El-Serag *et al.*, 1996) have been suggested as being involved in the pathogenesis of Crohn's disease. In order to elucidate this multifactorial aetiology and facilitate research into the complex interactions between the environment, immune system and genetic background, numerous experimental models of IBD have been developed (reviewed by Warren and Watkins, 1994; Elson *et al.*, 1995).

Although none of these animal models exactly reproduces the human inflammatory bowel diseases, the data already obtained allow certain conclusions to be made. In mice, an increased susceptibility to intestinal inflammation is associated with defects in numerous genes including the T cell receptor gene (Mombaerts *et al.,* 1993), the interleukin-10 gene (Kühn *et al.,* 1993), the interleukin-2 gene (Sadlack *et al.,* 1993) and the Gα-i2 gene (Rudolph *et al.,* 1995). There is also convincing evidence that intestinal bacteria stimulate enteritis in these models (Kühn *et al.,* 1993; Rath *et al.,* 1996; Dianda *et al.,* 1997). This is consistent with previous study in which the postileostomy reccurrence of Crohn's disease has been observed in a large proportion of patients receiving unfiltered ileostomy contents, but not in patients receiving a 22nm ultrafiltrate, from which most of conventional bacteria had been excluded (Harper *et al.,* 1985).

Crohn's disease bears some resemblance to paratuberculosis and is characterised by granulomatous lesions typically concentrated in the terminal ileum (Dalziel, 1913). Apart from the intestines, the upper alimentary tract (Krisner, 1991) and occasionally the nasal cavity may be involved (Kinnear, 1985). Both diseases usually affect young adults. The similarities between lesions of Crohn's disease and those observed in paratuberculosis led to numerous investigations for mycobacteria as an infectious cause of the disease. In 1984, *M. paratuberculosis* was isolated, for the first time, from intestinal biopsies from a patient with the disease. This isolate, "Linda", was successfully used to infect infant goats and produce granulomatous enteritis and regional lymphadenitis (Chiodini *et al.,* 1984a). Subsequently, other laboratories have also isolated *M. paratuberculosis* from patients with Crohn's disease (Graham *et al.,* 1987; Gitnick *et al.,* 1989). In contrast to the strain "Linda", the strain 410 isolated by Gitnick *et al.* (1989) did not produce granulomatous enteritis in goats. The identification of *M. paratuberculosis* 410 strain was based on phenotype, not genotype, and may not be reliable, explaining its inability to cause paratuberculosis in goats. In these and other studies, slowly growing, cell wall-deprived, spheroplasts have also been isolated from a proportion of IBD cases and a smaller number of control tissue samples (Chiodini *et al.,* 1986; Graham *et al.,* 1987; Markesich *et al.,* 1988; Gitnick *et al.,* 1989). The spheroplasts could not be identified by conventional means, although some of these agents later transformed into *M. paratuberculosis* (Chiodini *et al.,* 1986; Gitnick *et al.,* 1989).

Recent advances in molecular biology, especially the identification of IS*900*, provided the opportunity to further identify the isolates from patients with Crohn's disease. The target DNA (IS*900*) has been detected in approximately one third of uncharacterised isolates from cases of Crohn's disease (Moss *et al.,* 1992a; Wall *et al.,* 1993). However, one spheroplast-like organism from a control tissue has also been identified as *M. paratuberculosis* (Wall *et al.,* 1993), while other uncharacterised isolates from both control tissues and Crohn's disease cases have been found to contain either *M. avium* DNA or mycobacterial DNA of unknown origin (Moss *et al.,* 1992a; Wall *et al.,* 1993).

In order to further elucidate the role of *M. paratuberculosis* in the pathogenesis of Crohn's disease, numerous investigators have applied IS*900* based PCR assays to fresh and fixed tissue specimens from patients with IBD. The reported detection rate of *M. paratuberculosis* DNA in tissue samples from Crohn's disease cases varies from 13% to 100% (Sanderson *et al.,* 1992; Dell'Isola *et al.,* 1994; Fidler *et al.,* 1994; Lisby *et al.,* 1994; Murray *et al.,* 1995; Suenaga *et al.,* 1995). The target DNA has also been detected in intestinal tissues of ulcerative colitis and non-IBD cases (Sanderson *et al.,* 1992; Dell'Isola *et al.,* 1994; Murray *et al.,* 1995; Suenaga *et al.,* 1995). However, others have found no evidence of *M. paratuberculosis* DNA in specimens from patients with Crohn's disease (Rowbotham *et al.,* 1995; Dumonceau *et al.,* 1996; Riggio *et al.,* 1997; Al-Shamali *et al.,* 1997). The results of these studies are inconclusive and possible explanations for the disparity between the reported findings include variations in the methodology used, or misclassification of samples. Similarly conflicting is the immunological evidence for a mycobacterial association with Crohn's disease (reviewed by Thompson, 1994).

The recognition of paratuberculosis in wild rabbits (Greig *et al.,* 1997) and in stumptail macaques (McClure *et al.,* 1987) indicate that *M. paratuberculosis* is capable of crossing species lines from ruminants to non-ruminants. The potential public health risk posed by the organism has recently been addressed by studies evaluating the efficacy of pasteurisation for elimination of *M. paratuberculosis* from dairy products. In one study, the use of a laboratory-scale pasteuriser unit has demonstrated that treatment of raw milk at 72 degrees C for 15 seconds effectively killed all *M. paratuberculosis* organisms (Stabel *et al.,* 1997). Others have found that the organism present in milk may not be completely inactivated by heat treatments simulating high-temperature, short-time

pasteurisation (HTST) (Chiodini and Hermon-Taylor, 1993; Grant *et al.,* 1996; Sung and Collins, 1998). The variation in thermal resistance of various strains of the organism (Chiodini and Hermon-Taylor, 1993) is a possible explanation for the discrepancy between reported results. The isolation *M. paratuberculosis* from retail pasteurised cows milk supports the premise that the organism may survive HTST pasteurisation (Millar *et al.,* 1996).

The association between *M. paratuberculosis* and Crohn's disease remains controversial. A growing body of evidence suggests that genetic factors play a permissive role while the cause-effect relationship between chronic enteritis and a variety of initiating agents has not been established. Data derived from experimental models of IBD indicate that multiple pathways lead to chronic enteritis (Elson *et al.,* 1995; Zumla and James, 1996). Thus, the possibility of association between *M. paratuberculosis* and certain subcategories of IBD must be considered. Confirmation of such an association would have considerable public health implications. Well-designed epidemiological studies are needed to determine the role of *M. paratuberculosis*, and possibly other mycobacterial species, in the aetiology and pathogenesis of IBD.

## 1.2. Aims of the thesis

The purpose of the present study was three-fold. Firstly, to determine whether *M. paratuberculosis* DNA can be detected by IS*900*-based PCR in various tissues of sheep in different stages of paratuberculosis. Secondly, to give an overall perspective on the currently available options of controlling the disease, and thirdly to broaden the knowledge of the immune response in paratuberculosis. The latter is important since better understanding of the complex host response to infection with *M. paratuberculosis* is the first step toward either improving immunodiagnostic tests or manipulating the immune response in favour of the host. The specific aims of the thesis were as follows:

(i) To develop, validate and optimise a PCR assay for the detection of *M. paratuberculosis* in tissue samples and to apply the PCR assay to clinical samples, including blood and liver biopsy specimens.

(ii) To develop an experimental model of paratuberculosis.

(iii) To compare the diagnostic performance of immunodiagnostic tests for paratuberculosis in sheep experimentally infected with *M. paratuberculosis.*

(iv) To determine the protective value of a live-attenuated vaccine in lambs already infected with *M. paratuberculosis.*

(v) To investigate the progress of the systemic immune response in sheep infected experimentally with *M. paratuberculosis.*

(vi) To assess antigen-induced production of gamma interferon in samples of peripheral lymph nodes from sheep infected experimentally with *M. paratuberculosis.*

# Chapter 2

# Development of a rapid assay based on polymerase chain reaction for the detection of *Mycobacterium paratuberculosis*

## 2.1. Introduction

The isolation of *Mycobacterium paratuberculosis* is difficult and time consuming due to the slow growth rate of the organism and the need for lengthy decontamination processing, taking months or even years for confirmation of identity (Chiodini *et al.*, 1986). Reducing the time required to identify the organism would allow a more rapid detection of infected animals and early institution of appropriate control measures. The advent of the polymerase chain reaction (PCR) and the identification of IS*900*, an atypical sequence considered unique for *M. paratuberculosis* (Green *et al.*, 1989) have provided an opportunity for rapid and accurate identification of this organism.

This chapter describes the development and optimisation of a PCR assay for detecting *M. paratuberculosis* in tissue samples. An assessment of the specificity and sensitivity of this assay is also presented.

## 2.2. Materials and methods

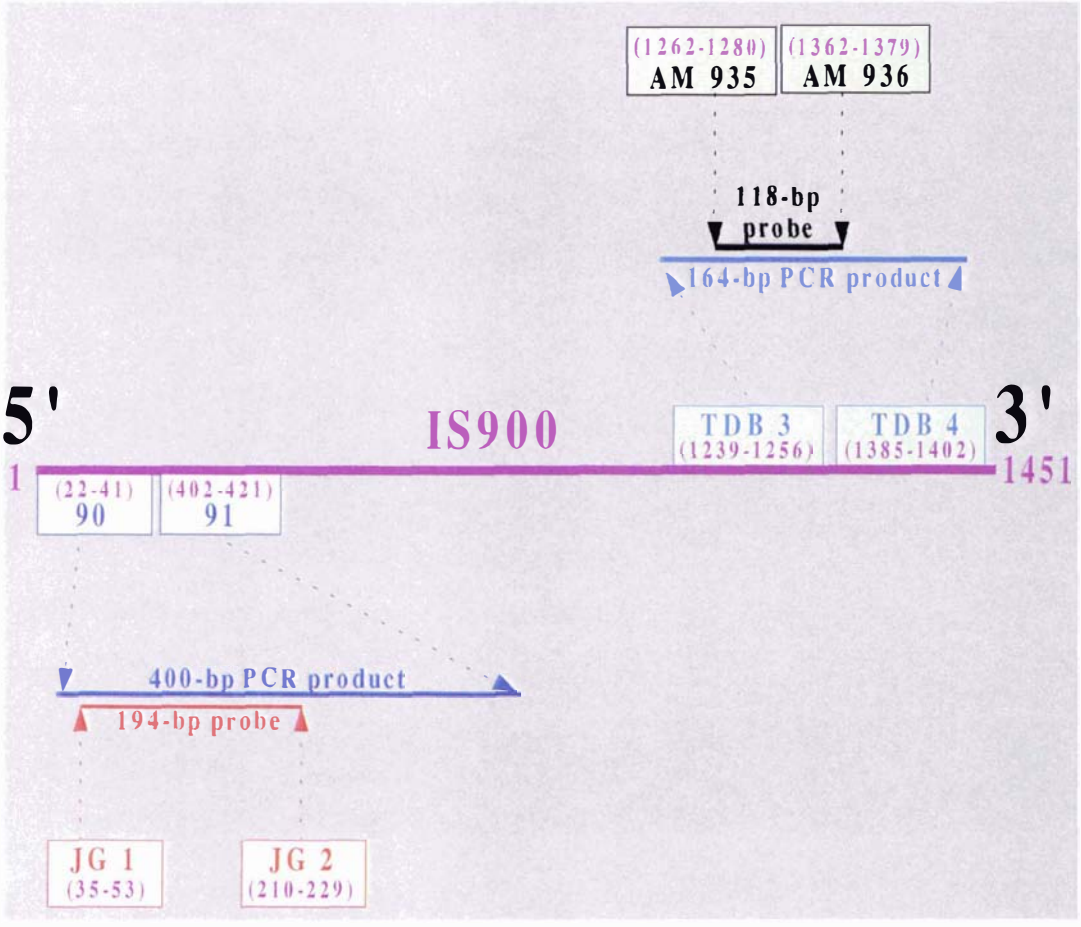
### 2.2.1. Oligonucleotide primer selection

Diagrammatic representation of primers used in this study is presented in Figure 2.1. Initially, primers TDB3 (5' GCG CCT GCT ACC TGT CGG 3') and TDB4 (5' GAC AGC GTC GTC GCG CAG 3') were used to amplify a 164-base pair (bp) fragment (Bassett, 1991). Internal primers AM935 (5' GTC AGC ATC CGC ACC GACA 3') and AM936 (5' CCC ACA GGA CGT TGA GGC 3'), described by Murray *et al.* (1995), were employed to produce a 118-bp fragment that served as a probe to confirm the

**Figure 2.1**. Diagrammatic representation of the regions of *M. paratuberculosis* insertion sequence IS*900* where primers 90/91, JG1/JG2, TDB3/TDB4 and AM935/AM936 are located. Figures in brackets represent location of primers in the IS*900* sequence.

identity of the 164-bp PCR products generated with primers TDB3 and TDB4. However, the TDB3 and TDB4 primers amplify a DNA fragment in the 3' region of the *M. paratuberculosis* IS*900* that shows high homology with the sequence of IS*901-902* from *M. avium* strains (Kunze *et al.*, 1991; Moss *et al.*, 1992b). Thus, alternative primers were adopted in this study.

**Figure 2.1.**




The primers 90 (5' GTT CGG GGC CGT CGC TTA GG 3') and 91 (5' GAG GTC GAT CGC CCA CGT GA 3'), previously described by Sanderson *et al.* (1992), were used to amplify a 400-bp fragment in the 5' region of IS*900*. Internal primers JG1 (5' GCT TAG GCT TCG AAT TGC C 3') and JG2 (5' CTC CGT AAC CGT CAT TGT CC 3') were designed and employed to produce a 194-bp fragment. This fragment served as a probe to confirm the identity of 400-bp PCR products generated with primers 90 and 91. The following primer selection criteria, published by Lowe *et al.* (1990), were adopted to design JG1 and JG2:

(i) The length of both primers should be 18-22 nucleotides.

(ii) If possible, primers should contain a GC-type sequence at their 3' end.

(iii) Each primer should have a similar GC content.

(iv) Primers should not contain more than four contiguous base pairs of homology to themselves or to each other, particularly at the 3' ends.

(v) Both primers should have comparable melting temperatures.

(vi) The amplification product should be less than 500-bp.

The melting temperature (Tm), at which oligonucleotides separate from complementary DNA sequence, was calculated for each primer using the following equation:

$$\mathbf{Tm = [2x(A+T)] \times [4x(G+C)]} \text{ (Bellamy, 1988)}$$

where A, T, G and C represent the number of adenine , thymine, guanine and cytosine nucleotides in the primer.

All primers were synthesised by the Centre for Gene Technology, University of Auckland, New Zealand, reconstituted in sterile distilled water to give a 400 μM concentration of each primer and stored at aliquots of 100 μl at -20°C until required.

## 2.2.2. Templates for amplification

### 2.2.2.1. DNA extraction from bacteria

For preparation of genomic bacterial DNA, the non-mycobacterial species listed in Table 2.1, were grown over 48 hours at 37°C on blood agar medium. Initially, the DNA was extracted from approximately 10 mg of bacterial growth using the GenePure™ 341 Nucleic Acid Purification system following the manufacturer's recommendations (Applied Biosystems Inc., USA).

Because the GenePure™ system was located in the laboratory where PCR products were analysed, and specificity problems were encountered initially, this automated method was replaced by a manual method of DNA extraction. The manual method was carried out in a class II bio-safety cabinet in a separate laboratory designated for this purpose.

**Table 2.1. Bacterial species and source of isolates or their DNA used in the assessment of the PCR specificity and sensitivity.**

| | Bacterial strains | Source |
|---|---|---|
| 1 | *Actinomyces pyogenes* | Clinical isolates obtained from the Veterinary Microbiology Laboratory, Massey University, Palmerston North, New Zealand |
| 2 | *Corynebacterium pseudotuberculosis* | |
| 3 | *Dermatophilus congolensis* | |
| 4 | *Escherichia coli* | |
| 5 | *Klebsiella pneumoniae* | |
| 6 | *Listeria monocytogenes* | |
| 7 | *Pasteurella haemolytica* | |
| 8 | *Proteus vulgaris* | |
| 9 | *Pseudomonas aeruginosa* | |
| 10 | *Rhodococcus equi* | |
| 11 | *Salmonella spp.* | |
| 12 | *Staphylococcus aureus* | |
| 13 | *Streptococcus agalactiae* | |
| 14 | *Streptococcus dysgalactiae* | |
| 15 | *Streptococcus uberis* | |
| 16 | *Yersinia pseudotuberculosis* | |
| 17 | *Mycobacterium africanum* | DNA kindly provided by Dr. A. Murray, Pathobiology Section, Massey University, Palmerston North, New Zealand |
| 18 | *Mycobacterium bovis (35725)* | |
| 19 | *Mycobacterium fortutitum* | |
| 20 | *Mycobacterium gordonae* | |
| 21 | *Mycobacterium intracellulare* | |
| 22 | *Mycobacterium marinum* | |
| 23 | *Mycobacterium microti* | |
| 24 | *Mycobacterium phlei* | |
| 25 | *Mycobacterium scrofulaceum* | |
| 26 | *Mycobacterium smegmatis* | |
| 27 | *Mycobacterium terrae* | |
| 28 | *Mycobacterium avium-intracellulare-scrofulaceum complex (5262)* | DNA kindly provided by Dr. G.W. de Lisle, Ag-Research, Wallaceville, New Zealand |
| 29 | *Mycobacterium avium-intracellulare-scrofulaceum complex (88-8805)* | |
| 30 | *Mycobacterium avium-intracellulare-scrofulaceum complex (58714)* | |
| 31 | *Mycobacterium paratuberculosis (ATCC 53950)* | Lyophilised strain kindly provided by Dr. A. Murray, Pathobiology Section, Massey University, Palmerston North, New Zealand |

***DNA extraction based on Proteinase K digestion and phenol-chloroform purification* (PC method)**

DNA was prepared by an adaptation of the method described by Ausubel *et al.* (1995). Approximately 20 mg of each bacterial growth was resuspended in 0.6 ml of buffer [100 mM NaCl, 25 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, 0.5% sodium dodecyl sulphate (SDS)] before adding 200 µg (10 µl of 20 mg/ml) of Proteinase K (Boehringer Mannheim, Germany). The mixture was incubated at 50°C overnight and then 100 µl of 3 M NaCl was added. The digest was mixed with an equal volume of 25:24:1 phenol:chloroform:isoamyl-alcohol and centrifuged at 15,800 g for 4 min before 0.6 ml of aqueous phase was collected and DNA precipitated with 0.9 ml of 100% isopropanol. After overnight storage at -20°C, the DNA was pelleted by centrifugation at 15,800 g for 15 min at 4°C and finally washed with 1.5 ml 70% ethanol and air-dried at 42°C. The DNA was eluted in 100 µl of sterile distilled water and stored at -20°C for subsequent use. Aliquots (10 µl) of the extracted DNA were examined following electrophoresis in 1% agarose gel in Tris-borate EDTA buffer (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA). The gels were stained with ethidium bromide and viewed using a 312 nm UV Spectroline TVC-312A transilluminator (Spectronics Corporation, USA). The concentration of extracted DNA was assessed spectrometrically and/or by visualisation of ethidium bromide-stained DNA on a gel.

*M. paratuberculosis* DNA was initially extracted by the same method used for all the other bacterial species. However, at the later stages of the PCR optimisation process, a further purification step using cetyltrimethylammonium bromide - CTAB (Aldrich Chemical Company, USA) was introduced. This compound has the ability to bind polysaccharides and proteins which may interfere with many enzymes and has previously been reported to improve the efficiency of the PCR for the detection of *M. bovis* (Wards *et al.*, 1995).

***DNA extraction based on Proteinase K digestion and CTAB-phenol-chloroform purification* (CTAB method)**

The protocol of DNA extraction using Proteinase K digestion and CTAB-phenol-chloroform purification was as follows: approximately 50 mg of lyophilised *M. paratuberculosis* was resuspended in 0.6 ml of buffer [100 mM NaCl, 25 mM EDTA pH 8.0, 10 mM Tris-HCl p H8.0, 0.5% SDS] before adding 200 µg (10 µl of 20

mg/ml) of Proteinase K (Boehringer Mannheim, Germany). The mixture was incubated at 50°C overnight and then 100 µl of 5 M NaCl and 80 µl of 10% CTAB in 0.7% NaCl was added. The digest was mixed with an equal volume of 25:24:1 phenol:chloroform:isoamyl-alcohol and centrifuged at 15,800 g for 4 min before 0.5 ml of aqueous phase was collected and DNA precipitated with 1.0 ml of 100% isopropanol. After overnight storage at -20°C, the DNA was pelleted by centrifugation at 15,800 g for 15 min at 4°C and finally washed with 1.5 ml 70% ethanol and air dried at 42°C. The DNA was eluted in 100 µl of sterile distilled water and 20 µl aliquots stored in -20°C for subsequent use.

### 2.2.2.2. DNA extraction from ovine solid tissues

In order to assess the suitability of various methods of PCR template preparation from mammalian tissues, the DNA was extracted from a segment of intestine from a sheep with histologically confirmed paratuberculosis, and from liver samples of 2 sheep which had no histological evidence of paratuberculosis. The animals were killed by intravenous injection of pentobarbitone and samples of tissues for PCR were collected immediately after euthanasia using sterile instruments and containers. Two methods of mammalian DNA preparation were assessed: simple boiling of tissue specimens and Proteinase K digestion followed by CTAB-phenol-chloroform extraction.

***Boiling method***

The sample of tissue was weighed, transferred to a stomacher bag (Seward, UK) and sterile distilled water was added in the proportion of 1.0 ml of water/100 mg of tissue. After homogenisation in a stomacher for 15-20 min, 1.0 ml of the homogenate was transferred to an Eppendorf tube and boiled for 30 min. The boiled homogenate was centrifuged at 15,800 g for 5 min and 20-30 µl of supernatant (equivalent of approximately 2-3 mg of tissue) was used in the PCR.

***Proteinase K digestion/CTAB-phenol-chloroform extraction method***

This method of DNA extraction was based on a protocol described by Ausubel *et al.* (1995). The sample of tissue was weighed, transferred to a stomacher bag and buffer [100 mM NaCl, 25 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, 0.5% SDS] added in

the proportion of 1.0 ml of buffer/100 mg of tissue. The tissue was then homogenised in a stomacher for 15-20 min and 0.7 ml of the homogenate transferred to an Eppendorf tube, before adding 200 µg (10 µl of 20 mg/ml) of Proteinase K and incubating at 50°C overnight. Subsequent to the incubation with Proteinase K, 115 µl of 5 M NaCl and 93 µl of 10% CTAB in 0.7% NaCl were added to 0.7 ml of the tissue digest, mixed well, and incubated at 65°C for 10-30 min. After cooling for 5 min at room temperature, the digest was mixed with 0.6 ml of chloroform, centrifuged at 15,800 g for 4 min, and 0.7-0.75 ml of aqueous phase was transferred to a fresh tube. This was mixed with an equal volume of 25:24:1 phenol:chloroform:isoamyl-alcohol and recentrifuged before 0.6 ml of aqueous phase was collected and DNA precipitated with 0.8 ml of 100% isopropanol. After overnight or longer storage at -20°C, the DNA was pelleted by centrifugation at 15,800 g for 15 min at 4°C, resuspended in 0.4 ml 0.3 M sodium acetate (pH 5.2) (BDH, England), precipitated with 1.2 ml absolute ethanol, repelleted, and finally washed with 0.5 ml of 70% ethanol and air dried at 42°C. The extracted DNA (from approximately 48 mg of intestine) was eluted in 120 µl of sterile distilled water and 5-7 µl (equivalent of approximately 2-3 mg of tissue) was used in the PCR.

### 2.2.3. PCR reaction mixture

***Taq DNA polymerase system***

During optimisation of the PCR, the reaction mixture contained 2 µM of each primer (90 and 91), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Germany) in a total volume of 50 µl.

In addition, following the completion of optimisation procedures and determination of the PCR detection limit and specificity, the suitability of the Expand Long Template amplification systems (Boehringer Mannheim, Germany) for the detection of *M. paratuberculosis* DNA was also assessed.

***Expand Long Template system***

The Expand Long Template System reaction mixtures were as follows:

(i) *Expand Long Template Buffer* I - 2 µM of each primer (90 and 91), 50 mM Tris-HCl

(pH 9.2), 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 0.35 mM of each dNTP, and 2.625 units of Taq/Pwo polymerase mixture in a total volume of 50 µl.

(ii) *Expand Long Template Buffer* II - 2 µM of each primer (90 and 91), 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.5 mM of each dNTP, and 2.625 units of Taq/Pwo polymerase mixture in a total volume of 50 µl.

(iii) *Expand Long Template Buffer* III - 2 µM of each primer (90 and 91), 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.5 mM of each dNTP, 2%(v/v) DMSO, 0.1% Tween® 20 and 2.625 units of Taq/Pwo polymerase mixture in a total volume of 50 µl.

Master mixes of all reagents were made and aliquots placed in 0.2 ml PCR reaction tubes to avoid reagent losses. Approximately 50 µl of sterile mineral oil (Sigma) was added to each tube to reduce evaporation. Tubes were kept on ice until template was added through the mineral oil. DNA amplification was conducted in a Perkin-Elmer Gene Amp 9600 cycler (Perkin-Elmer-Cetus, USA). The reactions were performed using sterile procedures and following contamination-free guidelines. Separate rooms, equipment, reagents and disposables were used for making up reagent mixtures, adding template, running the PCR and analysis of PCR products. The addition of template was conducted in a class II bio-safety cabinet (Gelman Science, Australia).

### 2.2.4. Generation and labelling of 194-bp probe

The JG1 and JG2 primers were employed to produce a 194-bp fragment that served as a template of a probe, which was used to confirm the identity of 400-bp PCR products generated with primers 90 and 91. The template of the probe was generated by the PCR. Briefly, approximately 2 ng of *M. paratuberculosis* DNA was added to a reaction mixture containing 2 µM of each primer (JG1 and JG2), 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 2.5 units of Taq polymerase (Boehringer Mannheim, Germany) in a total volume of 50 µl covered by mineral oil (Sigma, USA). The cycling conditions with the Perkin-Elmer Gene Amp 9600 were 1 cycle at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min with a 5 sec extension per cycle. After amplification, 15 µl aliquots of ampli-

fication mixture were electrophoresed in 2% agarose gel in TAE with molecular size markers (ΦX174 RF DNA/*Hae* III Fragments). The 194-bp band of ethidium bromide-stained DNA was visualised on a 312 nm UV transilluminator, carefully excised from the gel, and then transferred to an Eppendorf tube. The PCR product was purified using the GeneClean system according to the manufacturer's recommendations (BIO 101 Inc., USA). After elution in 20 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA), the concentration of the purified probe was estimated following electrophoresis of 3-5 µl of the probe in 2% agarose gel stained with ethidium bromide.

The purified probe was radioactively labelled using the RadPrime DNA labelling system (GIBCO BRL, USA). Approximately 1 ng of the purified probe was suspended in 22 µl of water and boiled for 4 min to denature the DNA. The probe was then rapidly cooled on ice and 15 µl of random primers, 6 µl of dNTP mixture, 3 µl of radioactive label ([$\alpha$-$^{32}$P] dCTP, 10 mCi/ml, ~3000 Ci/mmol (Amersham, UK) and 1 µl of Klenow DNA polymerase were added and incubated at room temperature overnight. Unincorporated nucleotides were removed using a NICK™ column (Pharmacia Biotechnology, Sweden) equilibrated with TE. The probe was eluted from the column with 400 µl of TE.

### 2.2.5. Validation of identity of 194-bp PCR product

After purification of 194-bp PCR products (generated by JG1 and JG2 primers) from a gel the sequencing of the amplicons was performed by the Massey University DNA Analysis Service.

### 2.2.6. PCR product analysis

After amplification, the analysis consisted of electrophoresis of PCR products in 2% agarose gel and then the identity of the amplicons generated by the 90 and 91 primers was confirmed by hybridisation with the radioactively labelled 194-bp probe.

***Electrophoresis of PCR products***

Aliquots (10 µl) of each amplification mixture were electrophoresed in 2% agarose gel in TBE. Molecular size markers (ΦX174 RF DNA/*Hae* III Fragments, GIBCO BRL,

USA) were run concurrently. The ethidium bromide-stained DNA bands were visualised on a 312 nm UV transilluminator, and photographed using Polaroid 667 film.

***Southern blot hybridisation***

After electrophoresis, the agarose gel was denatured in 0.5 M NaOH, 1.5 M NaCl for 45 min and neutralised in 1 M Tris-HCl pH 7.5, 1.5 M NaCl for 45 min. The agarose gel was then placed on top of two pieces of Whatmann 3MM chromatography paper saturated with 20xSSC (1xSSC is 0.15M NaCl, 15mM Na citrate; pH 7.0). A pre-wetted nylon membrane (Hybond-N, Amersham, UK) was placed over the gel and air bubbles carefully removed. Two pieces of chromatography paper were placed over the membrane, followed by a wad of absorbent tissues, and weighted down by a 0.5 kg weight. Transfer was allowed to occur overnight (approximately 16 h). The membrane was washed with 2xSSC and the DNA immobilised by ultraviolet light irradiation for 3.5 min.

The blot was hybridised, with constant shaking, at 65°C for 2 h in Rapid-hyb buffer (Amersham, UK) containing the radioactively labelled 194-bp probe (JG1/JG2 PCR product ). After hybridisation, the membrane was washed under stringent conditions; once in 2xSSC, 0.1% SDS at room temperature for 10 min, followed by one wash in 1xSSC, 0.1% SDS at 65 °C for 10 min and finally in 0.7xSSC, 0.1% SDS at 65°C for 15 min. It was then autoradiographed overnight (approximately 16 h) at -70°C on Kodak X-Omat X-ray film with an intensifying screen (Cronex, DuPont).

## 2.2.7. Optimisation procedures

The sensitivity and specificity of the PCR are affected by magnesium concentration, temperature and time of denaturation, annealing and extension steps, and the number of amplification cycles (Steffan and Atlas, 1991).

***Magnesium concentration***

Magnesium concentrations in the reaction were increased by the addition of sterile $MgCl_2$ (Mg). For the JG1 and JG2 primers (at 1 and 2 μM) the following concentrations of Mg were tested: 0.5, 1.0, 1.5, 2.5, and 3.5 mM. The concentrations of Mg tested

with the 90 and 91 primers (at 1 and 2 μM) were as follows: 1.5, 2.5, 3.0, 3.5 mM. Approximately 2 ng of *M. paratuberculosis* DNA was used as the template in each reaction.

***Number of cycles, times and temperatures of denaturation, annealing and extension***

For the 90 and 91 primers, a standard temperature of 94°C was used for denaturation. Initially, a temperature of 55°C was chosen for annealing, however, this was increased to 62°C in order to eliminate non-specific priming during PCR. Primer extension was performed at a standard 72°C. The number of cycles and the times of denaturation, annealing and extension that were used during the optimisation of the PCR assay are presented in Table 2.2. The half-life of Taq DNA polymerase is 40 min at 95°C (Steffan and Atlas, 1991). In order to preserve high enzymatic activity throughout the PCR process, 1-min times of denaturation, annealing and extension were reduced by half after the first 5 cycles when the number of cycles was increased from 40 to 45.

***Nested PCR***

In order to increase the sensitivity of the PCR, the internal primers, JG1 and JG2, were used in a nested PCR. The initial amplification of the template was conducted with primers 90 and 91 in a one-phase PCR at 55°C annealing temperature and then 1 μl of the first reaction mixture was reamplified in a second reaction mixture containing 2 μM of each primer (JG1 and JG2), 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1.25 units of Taq polymerase in a total volume of 25 μl covered by mineral oil.

The cycling conditions with the Perkin-Elmer Gene Amp 9600 were 1 cycle at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min with a 5 sec extension per cycle. The nested PCRs were performed using sterile procedures and following contamination-free guidelines as before. In addition, during the transfer of the first PCR product for the second amplification, separate pairs of disposable gloves and aerosol barrier pipette tips were used between each sample.

**Table 2.2. Amplification conditions evaluated during the process of optimisation of the PCR with the 90 and 91 primers.**

| PCR code | PCR cycling conditions (Temperature / Time / Number of cycles) | | | | | | | | | Total number of cycles |
|---|---|---|---|---|---|---|---|---|---|---|
| | Initial denaturation | First phase | | | | Second phase | | | | |
| | | Denaturarion | Annealing | Extension | Number of cycles | Denaturarion | Annealing | Extension | Number of cycles | |
| I | 94*/4 min (1 cycle) | 94*/1 min | 55*/1 min | 72*/1 min + 5 sec^ | 35 | na | na | na | na | 35 |
| II | 94*/4 min (1 cycle) | 94*/1 min | 62*/1 min | 72*/1 min + 5 sec^ | 35 | na | na | na | na | 35 |
| III | 94*/4 min (1 cycle) | 94*/1 min | 62*/1 min | 72*/1 min + 5 sec^ | 40 | na | na | na | na | 40 |
| **Standard** | 94*/4 min (1 cycle) | 94*/1 min | 62*/1 min | 72*/1 min | 5 | 94*/30 sec | 62*/30 sec | 72*/30 sec + 5 sec^ | 40 | 45 |

* degrees Celsius
^ additional extension time added per each cycle
**na** not applicable

**Figure 2.2.** Optimisation of concentrations of magnesium and primers JG1 and JG2 with the Taq DNA polymerase system.

**Lane number**:

**0**-molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1**-0.5 mM Mg, **2**-1.0 mM Mg, **3**-2.5 mM Mg, **4**-3.5 mM Mg, **5**-1.5 mM Mg (commercial buffer), **6**-1.5 mM Mg (own preparation), **7**-0.5 mM Mg, **8**-1.0 mM Mg, **9**-2.5 mM Mg, **10**-3.5 mM Mg, **11**-1.5 mM Mg (commercial buffer), **12**-1.5 mM Mg (own preparation).

### 2.2.8. Specificity of the PCR

The specificity of the PCR (defined as the ability of the PCR to amplify only DNA from *M. paratuberculosis*) was assessed using approximately 2 ng of bacterial genomic DNA as a template in a standard PCR. Species of bacteria used in the present study and their source are presented in Table 2.1.

### 2.2.9. Sensitivity of the PCR

The *in vitro* detection limit, or sensitivity, of the PCR (defined as the lowest quantity of template detectable in the assay) was assessed using DNA extracted from *M. paratuberculosis*. Starting with a known quantity of DNA, estimated visually on a gel against DNA mass ladder (GIBCO BRL, USA), ten-fold serial dilutions were made from approximately 0.5 ng to 0.5 fg and amplified under PCR cycling conditions presented in Table 2.2. The "*in vivo*" detection limit was determined by spiking ovine liver DNA, an equivalent of approximately 5 mg of tissue, with ten-fold serial dilutions of *M. paratuberculosis* DNA.

## 2.3. Results

### 2.3.1. Optimisation of magnesium concentration and sensitivity of the PCR

The optimum magnesium concentration for both sets of primers (90/91 and JG1/JG2) was shown to be 1.5 mM, the concentration in the commercial buffer supplied with the Taq polymerase (Figures 2.2 and 2.3).

**Figure 2.2.**

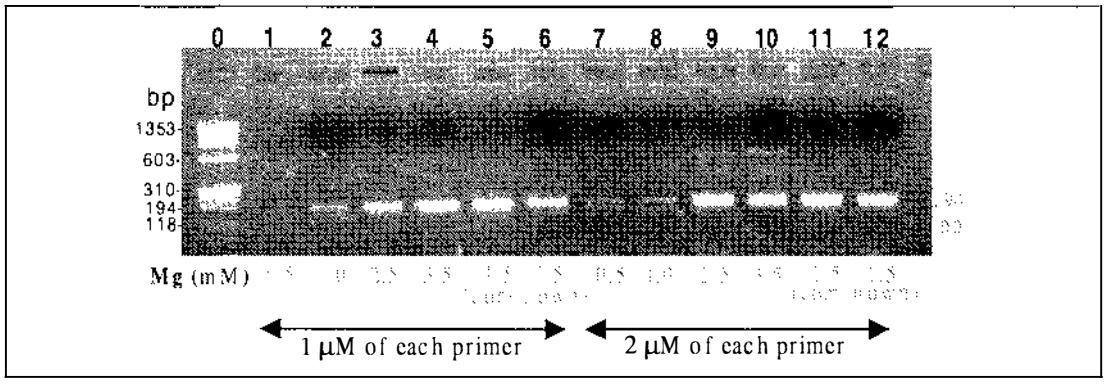


**Figure 2.3.** Optimisation of concentrations of magnesium and primers 90 and 91 with the Taq DNA polymerase system.

**Lane number**:

**0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1** - 1.5 mM Mg (commercial buffer) + 1 μM of each primer, **2** - 1.5 mM Mg (commercial buffer) + 2 μM of each primer, **3** - 2.5 mM Mg + 1 μM of each primer, **4** - 2.5 mM Mg + 2 μM of each primer, **5** - 3.0 mM Mg + 1 μM of each primer, **6** - 3.0 mM Mg + 2 μM of each primer, **7** - 3.5 mM Mg + 1 μM of each primer, **8** - 3.5 mM Mg + 2 μM of each primer.

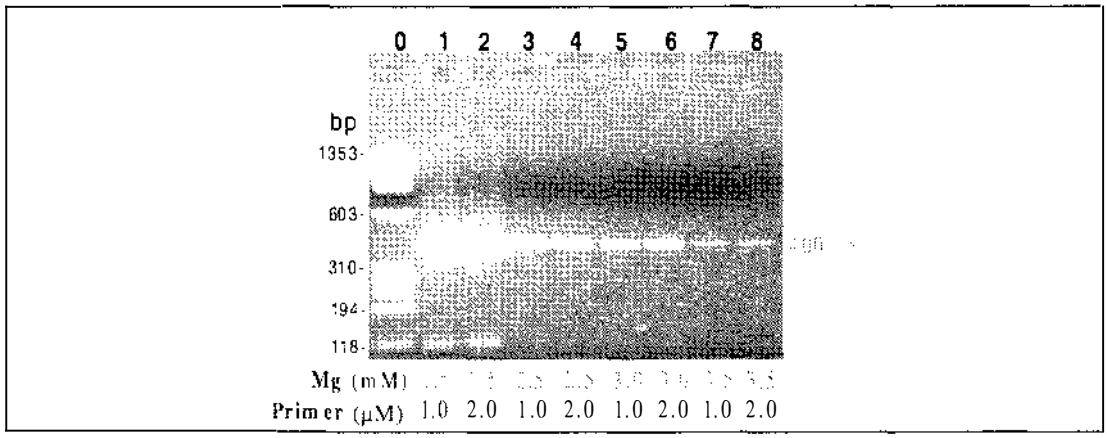
**Figure 2.4.1.** Sensitivity of the PCR (90 and 91 primers) with the Taq DNA polymerase system in a one phase PCR (constant 1-minute cycling conditions) at 35 (A) and 40 (B) cycles.

**Lane number**:

**A** **0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1** - reagent negative control, **2** - 5 pg of *M. paratuberculosis* DNA, **3** - 50 pg of *M. paratuberculosis* DNA, **4** - 500 pg of *M. paratuberculosis* DNA

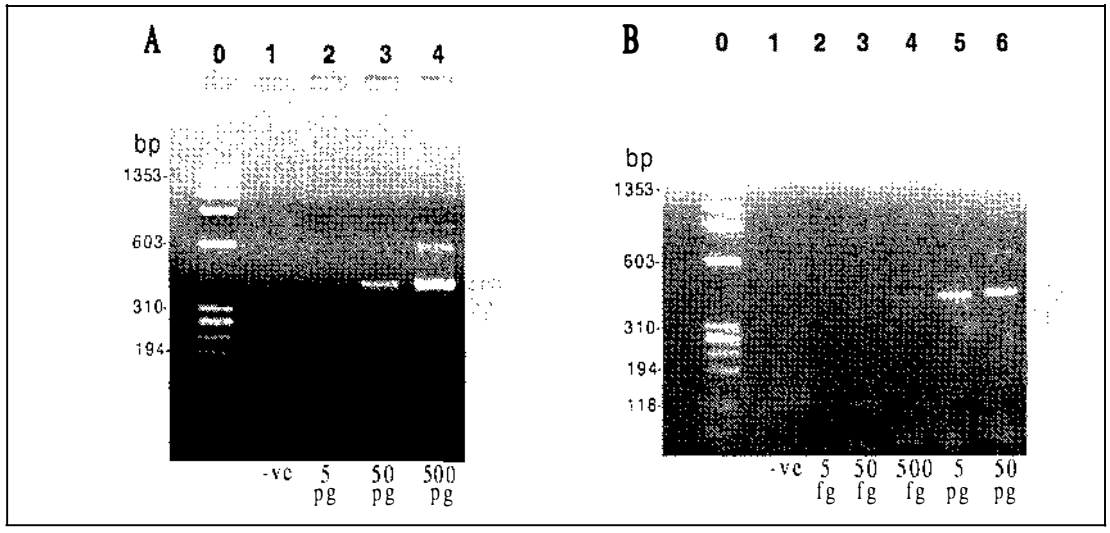
**B** **0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1** - reagent negative control, **2** - 5 fg of *M. paratuberculosis* DNA, **3** - 50 fg of *M. paratuberculosis* DNA, **4** - 500 fg of *M. paratuberculosis* DNA, **5** - 5 pg of *M. paratuberculosis* DNA, **6** - 50 pg of *M. paratuberculosis* DNA.

**Figure 2.3.**




The increase of the annealing temperature from 55°C to 62°C in a one-phase PCR (35 cycles with 90/91 primers) was not associated with significant difference in the assay's ability to detect the target DNA. There was a 100-fold increase in the detection limit, from 50 pg to 500 fg of *M. paratuberculosis* DNA (extracted by the PC method), when the number of cycles, in a one-phase PCR with 1-min times of denaturation (94°C), annealing (62°C) and extension (72°C), were increased from 35 to 40 (Figure 2.4.1).

**Figure 2.4.1.**




Further improvement of the assay sensitivity was noted when CTAB-purified DNA was used as a template in a standard two-phase, 45-cycle PCR in which 1-min times of denaturation, annealing and extension were reduced by half after the first 5 cycles. At these cycling conditions 1 fg of the CTAB-purified *M. paratuberculosis* DNA was detected (Figure 2.4.2). This detection limit was reproduced in 3 of 5 PCR runs. When

**Figure 2.4.2.** Sensitivity of the PCR (90 and 91 primers) with the Taq DNA polymerase system in a two-phase, 45-cycle PCR in which 1-minute times of denaturation (94°C), annealing (62°C) and extension (72°C) were reduced by half (30 seconds) after the first 5 cycles.

**Lane number**:

**A 0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1** - 5 pg of *M. paratuberculosis* DNA, **2** - 500 fg of *M. paratuberculosis* DNA, **3** - 50 fg of *M. paratuberculosis* DNA, **4** - 5 fg of *M. paratuberculosis* DNA, **5** - reagent negative control

**B 0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1** - 5 fg of *M. paratuberculosis* DNA, **2** - 1 fg of *M. paratuberculosis* DNA, **3** - 0.5 fg of *M. paratuberculosis* DNA, **4** - reagent negative control.

**Figure 2.4.3.** Sensitivity of the PCR on samples of ovine DNA spiked with *M. paratuberculosis* DNA in a two-phase, 45-cycle (Taq DNA polymerase) PCR system in which 1-minute times of denaturation (94°C), annealing (62°C) and extension (72°C) were reduced by half (30 seconds) after the first 5 cycles.

**Lane number**:

**0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments, **1** - 10 pg of *M. paratuberculosis* DNA, **2** - 1 pg of *M. paratuberculosis* DNA, **3** - 100 fg of *M. paratuberculosis* DNA, **4** - 10 fg of *M. paratuberculosis* DNA, **5** - 1 fg of *M. paratuberculosis* DNA, **6** - reagent negative control.

ovine DNA, an equivalent of 5 mg of tissue, was spiked with serial dilutions of *M. paratuberculosis* DNA the *"in vivo"* detection limit was 10 fg of target DNA (Figure 2.4.3).

No improvement in sensitivity was observed with nested PCR and, despite extensive precautions, uncontrollable specificity problems were encountered.

**Figure 2.4.2.**

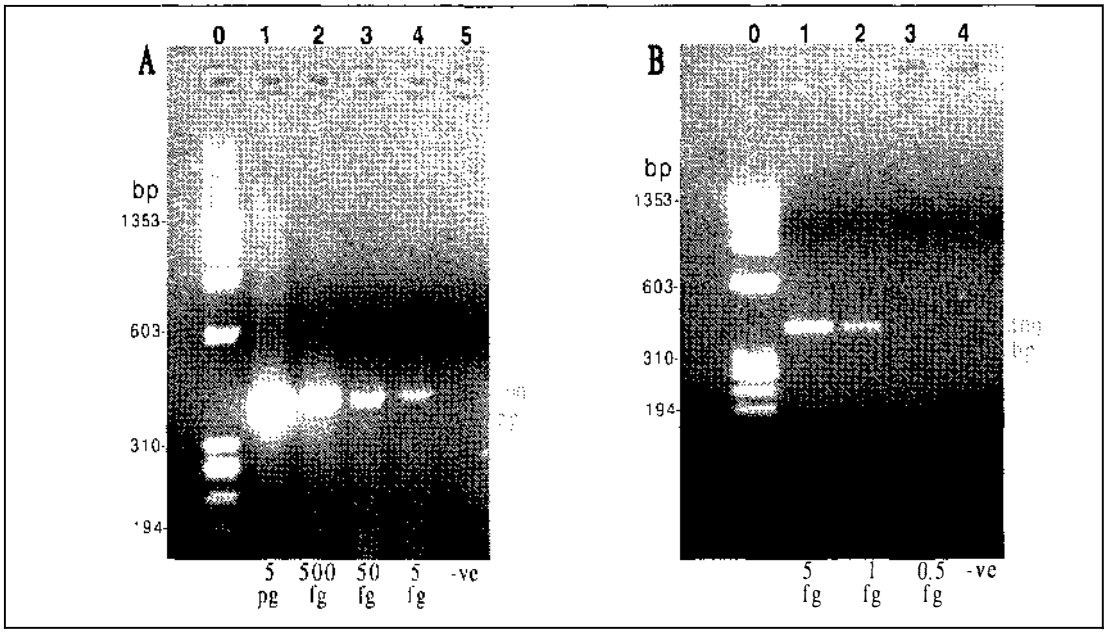
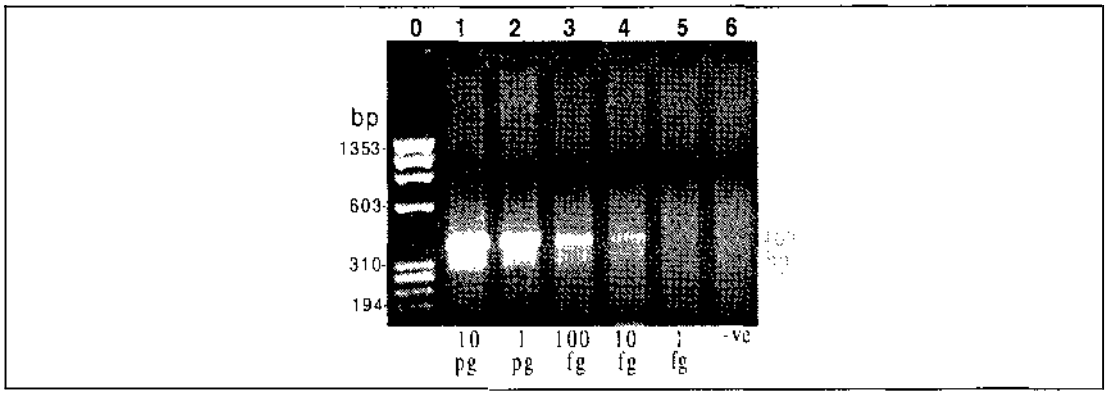



**Figure 2.4.3.**




## 2.3.2. Specificity of the PCR

Of 30 bacterial species tested, only *M. scrofulaceum* DNA showed cross-reactivity with 90 and 91 primers (Figure 2.5).

**Figure 2.5.** Specificity of the PCR. Separation of 400-bp PCR products (generated by primers 90 and 91) on 2% gel (A) and autoradiography of southern transfer blot (B) following hybridisation of the 400-bp PCR products with 194-bp radioactively-labelled probe (generated by primers JG1 and JG2).

**Lane number**: **0** - molecular size marker (ϕX174 RF DNA/*Hae* III Fragments), **1** - *M. phlei*, **2** - *M. bovis* (35725), **3** - *M. gordonae*, **4** - *M. smegmatis*, **5** - *M. terrae*, **6** - *M. microti*, **7** - *M. marinum*, **8** - *M. fortutitum*, **9** - *M. africanum*, **10** - *M. scrofulaceum*, **11** - *M. intracellulare*, **12** - *M. avium* - *intracellulare* - *scrofulaceum* complex (88-8805), **13** - *M. avium* - *intracellulare* - *scrofulaceum* complex (5262), **14** - *M. avium* - *intracellulare* - *scrofulaceum* complex (58714), **15** - reagent negative control, **16** - *M. paratuberculosis* (positive control).

**Figure 2.6.** Comparison of PCR yields achieved in samples of ovine DNA extracted by boiling and by the Proteinase K digestion/CTAB-phenol-chloroform extraction method.

**Lane number**:

**0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments,

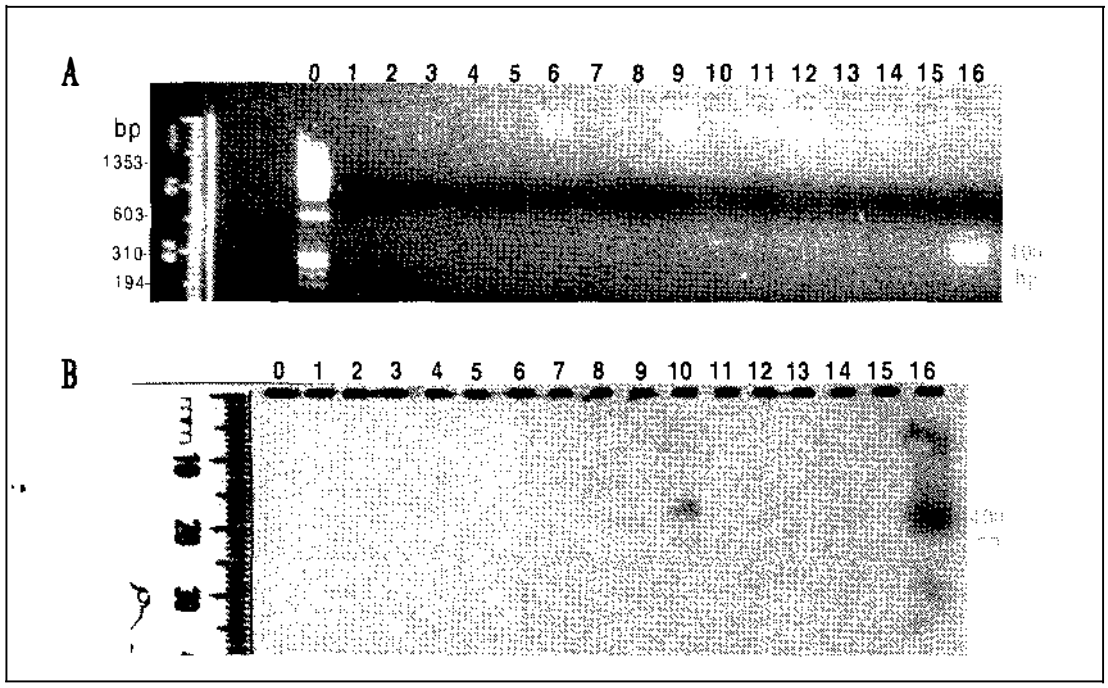
**1 to 3** - DNA extracted from liver of a sheep without histological evidence of paratuberculosis by boiling (equivalent of approximately 3 mg tissue),

**4 to 6** - DNA extracted from liver of a sheep without histological evidence of paratuberculosis by the Proteinase K digestion/CTAB-phenol-chloroform extraction method (equivalent of approximately 3 mg tissue),

**7 and 8** - DNA extracted from intestine of a sheep with histologically confirmed paratuberculosis by boiling (equivalent of approximately 2 mg tissue),

**9 and 10** - DNA extracted from intestine of a sheep with histologically confirmed paratuberculosis by the Proteinase K digestion/CTAB-phenol-chloroform extraction method (equivalent of approximately 2 mg tissue)

**Figure 2.5.**




## 2.3.3. Methods of DNA extraction from mammalian tissues

Of the two methods used to extract DNA from samples of intestine from a sheep with histologically confirmed paratuberculosis, the amplification of the template prepared by the Proteinase K digestion/CTAB-phenol-chloroform extraction method produced stronger signal (400-bp product) on a gel than that obtained with the template prepared from a comparable amount of tissue (2 mg) by the boiling method (Figure 2.6).

**Figure 2.6.**

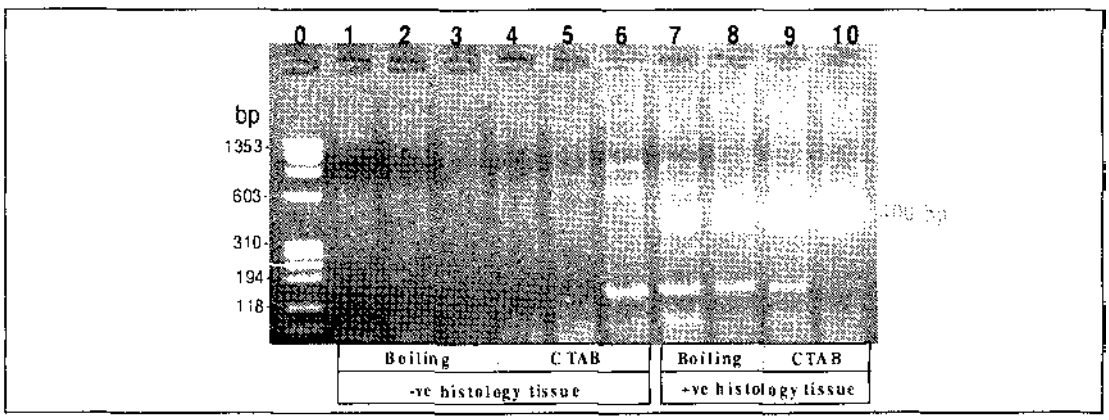


**Figure 2.7.** Comparison of the Taq DNA polymerase system with the Expand Long Template system.

**Lane number:**

**1 and 2** 10 fg *M. paratuberculosis* DNA + Taq

**3 and 4** 100 fg *M. paratuberculosis* DNA + Taq

**5 and 6** 1 pg *M. paratuberculosis* DNA + Taq

**7** 10 pg *M. paratuberculosis* DNA + Taq

**9 and 10** 10 fg *M. paratuberculosis* DNA + Expand Long Template

**11 and 12** 100 fg *M. paratuberculosis* DNA + Expand Long Template

**13 and 14** 1 pg *M. paratuberculosis* DNA + Expand Long Template

**15** 10 pg *M. paratuberculosis* DNA + Expand Long Template

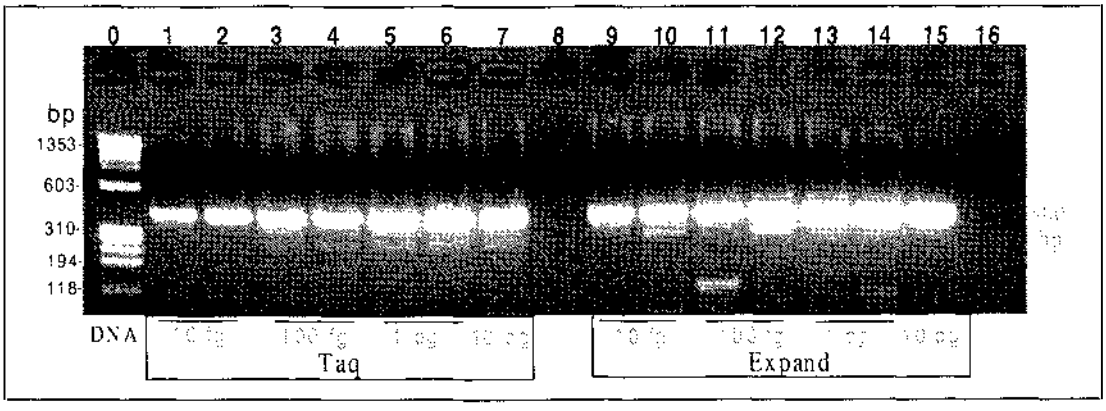
**8 and 16** no DNA

**0** - molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

### 2.3.4. Expand Long Template vs. Taq polymerase system

The optimum magnesium concentration for the 90 and 91 primers was shown to be 2.25 mM, the concentration in the commercial buffer II supplied with the Expand Long Template system. The comparison of the Taq polymerase system with the Expand Long Template system revealed that amplification of the target DNA using the latter system produced stronger signal (400-bp product) on a gel than that produced by Taq polymerase system (Figure 2.7).

**Figure 2.7.**




### 2.3.5. Validation of identity of 194-bp PCR product

The analysis of the sequence of 194-bp PCR product using the BLAST GenBank database program confirmed the identity of the PCR product generated by JG1 and JG2 primers.

## 2.4. Discussion

Theoretically, the PCR technique is able to detect a single copy of target DNA. In practice, however, such a detection level is rarely reached. This is due to both inherent PCR limitations (Sardelli, 1993) and inhibition of the amplification process by compounds co-precipitated with the DNA sample, either from the extraction reagents or from bacterial or mammalian cellular debris (Lo *et al.,* 1989; Mercier *et al.,* 1990; Rossen *et al.,* 1992; Folgueira *et al.,* 1993). One of the factors that affects the efficiency of PCR is the number of cycles (Bassett, 1991; Sardelli, 1993). In the

present study, increasing the number of cycles from 35 to 40 in a one-phase PCR with 1-min times of denaturation, annealing and extension was associated with an increase in the *in vitro* detection limit from 50 pg to 500 fg of *M. paratuberculosis* DNA. Further improvement of the assay sensitivity was noted when CTAB-purified DNA was used as a template in a standard two-phase, 45-cycle PCR in which 1-min times of denaturation, annealing and extension were reduced by half after the first 5 cycles. At these cycling conditions, 1 fg of the CTAB-purified *M. paratuberculosis* DNA was detected, a detection limit that is comparable with other studies in which CTAB was used for the extraction of DNA from *M. bovis* (Wards *et al.,* 1995).

A number of factors could have contributed to the improvement in PCR sensitivity. Firstly, it may have been due to the increase in the number of cycles. Secondly, shortening of the amplification steps could have led to higher and more constant activity of Taq polymerase throughout the amplification process. Thirdly, since CTAB has the ability to bind polysaccharides and proteins that may interfere with many enzymes (Ausubel *et al.,* 1995), potential PCR inhibitors may have been removed by this compound. Methods based on CTAB extraction have been favoured in the purification of DNA from bacteria (Ausubel *et al.,* 1995), and recently, the use of CTAB extraction from *M. bovis* has been reported to improve the efficiency of PCR (Wards *et al.,* 1995). The 1 fg detection limit was not reproducible with every PCR run. This is likely to be due to a sampling error that became obvious when the number of target DNA molecules in a test sample was low (Lo, 1994). In addition, there was a ten-fold decrease in the detection limit of the PCR when ovine DNA spiked with serial dilutions of *M. paratuberculosis* DNA was tested. This seems to exemplify the well-recognised inhibition of the amplification process by extraneous substances present in DNA samples.

The sensitivity of the PCR may be improved by reamplifying the initial PCR products using nested primers (Plikaytis *et al.,* 1990). In the present study, no improvement in sensitivity was observed with nested PCR, a finding that agrees with other studies (Wards *et al.,* 1995). Furthermore, despite extensive precautions, uncontrollable specificity problems were encountered. According to Weiss (1995), this is a common problem with nested PCR.

The specificity of the PCR is based on the number of false-positive results occurring. Such results commonly occur when lower annealing temperatures are used or when other organisms, which are present in samples tested, contain sequences of DNA that are complementary to the target sequence. Prior to optimisation of the PCR in this study, cross-reactions with various bacteria were seen. However, increasing the annealing temperature and manual extraction of DNA in a separate laboratory eliminated the problem. The PCR assay was found to be highly specific and among 30 bacterial genera tested only DNA from *M. scrofulaceum* showed cross-reactivity. Others have also found cross-reactions with *M. scrofulaceum* in IS*900*-based PCR and suggested that IS*900*-related sequences may occur in *M. scrofulaceum* (Thoresen and Olsaker, 1994). This possibility cannot be excluded since some strains show close phenotypic relatedness to *M. paratuberculosis* (Wasem *et al.,* 1991). Alternatively, the DNA sample of *M. scrofulaceum* used in the current study might have been contaminated with *M. paratuberculosis* DNA. Due to limited time and resources, the origin of the cross-reactivity with this organism was not investigated further. *M. scrofulaceum* is not considered to be a primary pathogen although it has occasionally been isolated from human patients with AIDS (Campos-Herrero *et al.,* 1996; Horsburgh, 1996), children with lymphadenitis (Pang, 1992; Turneer *et al.,* 1994) and immunocompromised mice (Ueda *et al.,* 1992). To our knowledge, this organism has never been isolated from sheep, and the possibility of *M. scrofulaceum* causing false positive results in clinical samples from sheep seems remote.

The boiling method for extraction of DNA from tissues has the advantage of speed and convenience. In contrast, the method of template preparation that involves Proteinase K digestion of tissue, followed by CTAB-phenol-chloroform purification, is tedious and protracted. Of these 2 methods, the amplification of the template prepared in the latter manner produced a stronger signal (400-bp product) on a gel than that obtained with the DNA prepared by the boiling method. This suggests a decrease in the sensitivity of the PCR when it is applied to crude DNA extracts and is in agreement with other studies (Wards *et al.,* 1995; Fenwick, 1997). Possible explanations include inadequate extraction of *M. paratuberculosis* from tissues by boiling, insufficient extraction of DNA from *M. paratuberculosis*, the presence of PCR inhibitors, or a combination of these.

The objective of the present study was to design a PCR assay for the detection of *M. paratuberculosis* in clinical material. This was successful and the application of the PCR assay to tissue samples from sheep with clinical paratuberculosis is presented in Chapter 3.

# Chapter 3

# Validation of the polymerase chain reaction assay for the detection of *Mycobacterium paratuberculosis* on samples of solid tissue and blood from sheep with clinical paratuberculosis

## 3.1. Introduction

Attempts to control paratuberculosis have been hampered by a lack of suitable diagnostic tests. Since seroconversion occurs relatively late in the course of infection (Chiodini *et al.,* 1984b; Cocito *et al.,* 1994.), the use of serological tests in the diagnosis of paratuberculosis is predominantly restricted to the diagnostic confirmation of suspected clinical cases and screening of herds for freedom from infection. Although culture of *Mycobacterium paratuberculosis* provides the definitive diagnosis of infection, the prolonged isolation time and difficulty to grow some strains (Gunnarsson, 1979; Brooks *et al.,* 1988; Shulaw *et al.,* 1993; Collins *et al.,* 1993a) limit the use of this diagnostic method especially in sheep. The attempts to apply polymerase chain reaction (PCR) for detection of *M paratuberculosis* in faeces have not been encouraging (Sockett *et al.,* 1992; van der Giessen *et al.,* 1992; Collins *et al.,* 1993a; Collins *et al.,* 1993b), presumably due to presence of inhibitory substances in faecal samples (Vary *et al.,* 1990; van der Giessen *et al.,* 1992; Collins *et al.,* 1993b).

This chapter describes the application of the PCR assay to blood and other tissues from sheep with clinical paratuberculosis and compares the results with those derived from histology and serology. The objective of the study was to validate the PCR assay for the detection of *M. paratuberculosis* in clinical specimens.

## 3.2. Materials and methods

### 3.2.1. Animals

Twenty ewes, aged between two and four years, were obtained from six farms with an established history of paratuberculosis. All sheep were in poor condition and were suspected of having Johne's disease. Ten clinically normal sheep, aged 14 months, were obtained from a farm on which paratuberculosis had not previously been diagnosed. These animals were used as negative controls

### 3.2.2. Sample collection

Prior to euthanasia, blood was collected from the jugular vein into 4.0 ml evacuated plain tubes for serology and into 4.0 ml tubes containing ethylenediaminetetra-acetic acid disodium (EDTA) anticoagulant for PCR. In order to reduce the risk of contamination of samples for PCR assay, blood was always drawn first into the plain tubes.

The animals were killed by intravenous injection of pentobarbitone. Immediately after euthanasia, and prior to necropsy, samples of tissues for PCR were collected using sterile instruments and containers.

### 3.2.3. Processing of samples and DNA extraction from tissues and blood

***Solid tissues***

DNA was extracted from samples of ileum, ileocaecal lymph node and liver using the Proteinase K digestion/CTAB-phenol-chloroform extraction method as described in Chapter 2 (2.2.2.2).

***Blood***

The whole blood samples (4.0 ml) were transferred to 15.0 ml Falcon tubes containing 10.0 ml of erythrocyte lysis buffer [0.16 M $NH_4Cl$, 10 mM $NaHCO_3$, 0.1 mM EDTA; pH 7.2] and incubated for 10 min at room temperature. After lysis of red blood cells, leukocytes were pelleted by centrifugation for 10 min at 2,300 g. The supernatant was then discarded and the pellet washed with 10.0 ml phosphate buffered saline [PBS; 0.14 M NaCl, 8 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$; pH 7.2], recentrifuged for 5 min at 2,300 g, and the supernatant again discarded. The pellet was resuspended in buffer [100 mM NaCl, 25 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0), 0.5% sodium dodecyl sulphate] to a total volume of 1.5 ml before adding 400 µg of Proteinase K (Boehringer Mannheim, Germany) and incubating the mixture at 50°C overnight. After the incubation, 0.7 ml of the digest was transferred to an Eppendorf tube and either processed immediately for DNA extraction, or stored at -20°C. The DNA was extracted from 0.7 ml of the digest of blood using the same Proteinase K digestion/CTAB-phenol-chloroform extraction method that was used for solid tissue (described in Chapter 2).

Each sample of solid tissue DNA was eluted in 120 µl of water and in order to assess the effect of concentration of DNA on the amplification process, volumes of 10, 20, and 30 µl (approximate equivalents of 4, 8, and 12 mg of tissue, respectively) were tested by PCR. The leukocyte DNA was resuspended in either 60 or 120 µl of water and equivalents of approximately 0.5 ml and 1.0 ml of blood were tested by PCR.

## 3.2.4. Polymerase Chain Reaction

***Amplification***

The PCR was performed under standard conditions as described in Chapter 2 (2.2.3). Each sample of extracted DNA (equivalents of approximately 0.5 and 1.0 ml of blood, and 8 and 12 mg of solid tissue) was amplified in a reaction mixture containing 2 µM of each primer (90 and 91), 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 5.0 units of Taq polymerase (Boehringer Mannheim, Germany) in a total volume of 100 µl, covered by mineral oil. In addition, samples of

solid tissue DNA (equivalent of approximately 4 mg of tissue) were amplified using the Expand Long Template system (Boehringer Mannheim, Germany) in a reaction mixture containing 2 μM of each primer, 50 mM of Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.5 mM of each dNTP, and 2.625 units of Taq/Pwo polymerase mixture in a total volume of 50 μl, covered by mineral oil.

For each set of reactions, positive (*M. paratuberculosis* DNA) and negative (water and reagents) controls were included. The reactions were performed using sterile procedures and following contamination-free guidelines, as described in Chapter 2 (2.2.3). All primers were manufactured by the Centre for Gene Technology, University of Auckland, New Zealand.

***PCR product analysis***

The analysis consisted of electrophoresis and visual examination of PCR products on agarose gel, as described in Chapter 2 (2.2.6). The identity of PCR product was confirmed by dot blot hybridisation of amplification products with an internal 194 base pair (bp) probe. The probe was generated and labelled with [$\alpha$-$^{32}$P] dCTP, 10 mCi/ml, ~3000 Ci/mmol (Amersham, UK) using the method described in Chapter 2 (2.2.4). For dot blot hybridisation, amplification mixtures in each PCR tube were heated for 4 min at 94°C, immediately transferred on ice, and then 4 μl aliquots of each reaction mixture deposited manually onto Hybond-N membranes (Amersham, UK). The membranes were air-dried for approximately 15 min at room temperature, denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min, and then neutralised in 1 M Tris-HCl (pH 7.5), 1.5 M NaCl for 5 min. After DNA cross-linking by ultraviolet light irradiation for 3.5 min, the membranes were hybridised, washed and autoradiographed under conditions described in Chapter 2 (2.2.6).

***Interpretation of PCR results***

The samples were classified as positive if a band of the right size (400-bp) was identified on a gel, the hybridisation results were concordant, and no signal was obtained with the negative control. The samples were considered as suspicious if a signal was obtained by the dot blot analysis but the 400-bp band was not visible on a gel. It is likely that such signals on the dot blot were due to small amounts of PCR

products that were indiscernible on the gel. However, the samples contained large amounts of mammalian DNA and these signals, frequently very weak, could also represent non-specific binding of the probe to the mammalian DNA or processing artefacts. Spiking of mammalian DNA with serial dilutions of PCR products to provide a cut-off signal, at which samples positive on the dot blot but negative on the gel would have been classified in a dichotomous manner (positive/negative), was not attempted. As the amount of mammalian DNA varied between samples, it would be very difficult to make an adjustment for each sample.

### 3.2.5. Histology

After fixation in 10% buffered formol saline, tissues were routinely processed, embedded in paraffin and sectioned at 4 μm. Two sections of each tissue sample were prepared. One of the sections was stained with hematoxylin and eosin and the second by Ziehl-Neelsen's method. Additional histological sections of the ileum, ileocaecal lymph node and liver were prepared from sheep suspected of having clinical paratuberculosis when PCR results obtained in these sheep were not concordant with histopathology.

### 3.2.6. Serology

All serological testing was done in the Central Animal Health Laboratory, Upper Hutt, New Zealand. The complement fixation test (CFT), the gel diffusion test (AGID), the Central Animal Health Laboratory enzyme-linked immunosorbent assays (CAHL ELISA) and the "modified" Commonwealth Serum Laboratories (CSL) ELISA were performed as described by Hilbink *et al.* (1994) with two exceptions. The gel diffusion antigen was used in the CAHL ELISA after filtration through a 0.2 μm filter (Gelman Science, USA) and the protein-G peroxide conjugate was obtained from Zymed, San Francisco, USA.

The sensitivity and specificity of the serological tests and the PCR assay were determined using the method described by Sackett *et al.* (1985).

## 3.3. Results

### 3.3.1. Histology

Of the twenty ewes suspected of having clinical paratuberculosis, twelve had granulomatous lesions typical of paratuberculosis. The ileal and jejunal lamina propria and, in some cases, the submucosa of these sheep were diffusely infiltrated by macrophages, lymphocytes and eosinophils and contained occasional aggregates of neutrophils. Numerous acid-fast organisms (AFO's) were present in the macrophages in intestinal sections from these twelve animals. The serosal lymphatics of the ileum and jejunum were surrounded by a zone of mononuclear cells and contained nests of lymphocytes and macrophages laden with AFO's.

In addition, aggregates of macrophages were present in afferent lymphatics, cortices of the ileocaecal lymph nodes and scattered throughout the parenchyma of the liver. The number and size of these microgranulomas varied between animals. In some cases as few as ten mononuclear cells constituted a microgranuloma while in other cases the epithelioid cells were too numerous to count. AFO's were numerous in six lymph nodes, sparse in five and in one lymph node, there was no evidence of AFO's. In contrast, the AFO's in hepatic microgranulomas were not evident in seven animals, sparse in four and abundant in only one.

The remaining 8 ewes in poor condition, and all 10 clinically normal control animals, had no granulomatous lesions typical of paratuberculosis in sections of ileum, ileocaecal lymph node and liver. All sheep examined had evidence of intestinal parasitism, including moderate diffuse eosinophilic infiltration of the ileal and jejunal lamina propria, abundant globule leukocytes, and the presence of parasites, or their remnants, in the intestinal lumen and/or lamina propria.

### 3.3.2. Polymerase Chain Reaction

Figures 3.1 and 3.2 show results of the PCR on selected samples of solid tissues and blood from sheep suspected of having clinical paratuberculosis.

As presented in Table 3.1, the PCR assay for *M. paratuberculosis* DNA was positive in 87 of 126 samples (69%) from the 12 sheep with histologically confirmed paratuberculosis. Although a minimal increase in the detection level was observed with 4 mg tissue DNA using the Expand Long Template system, there was no significant variation of test accuracy with different quantities of solid tissue DNA from these 12 sheep. In contrast, there was a decrease in the detection rate from 66% to 33% when the PCR assay was applied to 1.0 ml of blood compared with 0.5 ml of blood.

The detection rate of a single test was 100% for ileum, and 45-70% for other tissues, using histology as the "gold standard". There was low reproducibility of results of the PCR on samples of liver, ileocaecal lymph node and blood, possibly reflecting a sampling error due to the low number of target molecules in the original sample. When the results of single tests on samples of comparable DNA concentrations (4 mg tissue/DNA in 50 µl reaction volume and 8 mg tissue/DNA in100 µl reaction volume) were interpreted in duplicate, sensitivity was calculated 72% for ileocaecal lymph node, 90% for liver, and 100% for ileum.

In addition, among 85 samples from the 8 sheep in poor condition but without histological evidence of paratuberculosis, six (2 samples of ileocaecal lymph node and 4 samples of blood) were tested positive by PCR. These 6 positive reactions were obtained in 4 of the 8 sheep. In spite of detailed examination of additional sections of the ileum, ileocaecal lymph node and liver from these sheep, no histological evidence of paratuberculosis was detected.

No positive or suspicious reactions were obtained in 107 samples from the 10 clinically normal control sheep, giving the PCR assay 100% specificity.

**Figure 3.1.** Results of the PCR on selected samples of hepatic, ileal and ileocaecal lymph node DNA, equivalents of approximately 4 mg of tissue, from 20 sheep suspected of having clinical paratuberculosis. Separation of 400-bp PCR products, generated by primers 90 and 91 in the Expand Long Template system, on 2% gel (A, B, C and D). Dot blot results following hybridisation of the 400-bp PCR products with 194-bp radioactively-labelled probe (E). Dots are labelled with the alphabetical code of the gel and numbers of gel's lanes. Samples in lanes A1, A2, A3, A5, A7, A9, A11, A12, A13, A14, B1, B3, B5, B6, B13, B14, B15 D1, D2, D3, D9, D10, D11, D13, D15, D16 and D17 were classified as positive. Bands of 400-bp PCR products were present in these lanes and results of dot blot analysis were concordant. Samples in lanes A10, D6 and D8 were classified as suspicious, as no bands of 400-bp PCR products were visible on the gels but hybridisation signals were obtained in the corresponding dots.

**x** - sample from a sheep that was not part of this study.

**Tissue**:

**i** - ileum

**LN** - ileocaecal lymph node

**L** - liver

**Histology**:

**+** presence of lesions consistent with paratuberculosis in the ileum

**-** absence of lesions consistent with paratuberculosis in the ileum

**PCR**:

**-ve** negative control (no DNA)

**+ve** positive control (*M. paratuberculosis* DNA)

**0** molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

**Figure 3.1.**

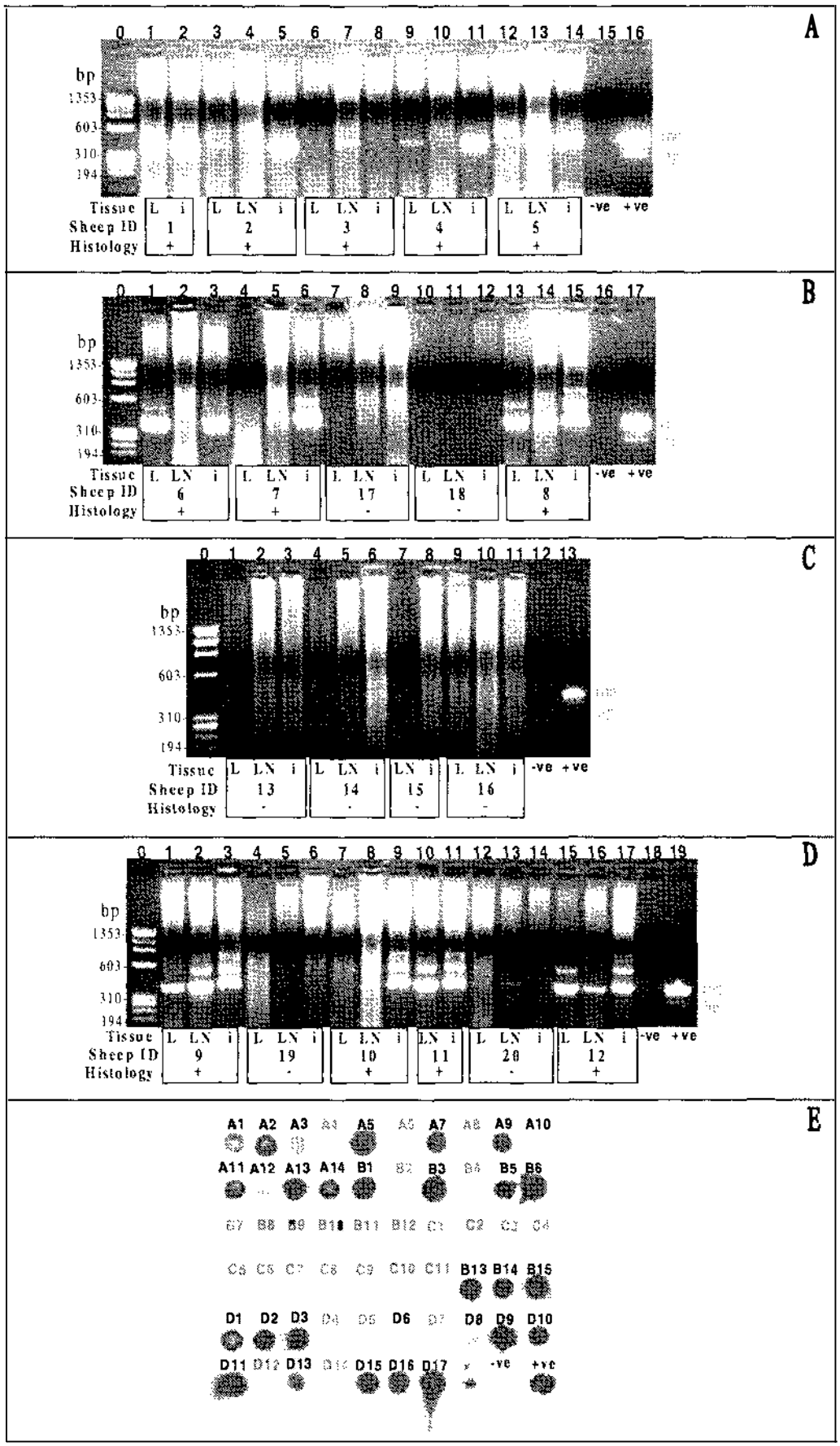

A
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
bp
1353
603
310
194
Tissue L i | L LN i | L LN i | L LN i | L LN i
Sheep ID 1 | 2 | 3 | 4 | 5
Histology + | + | + | + | +
-ve +ve
B
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
bp
1353
603
310
194
Tissue L LN i | L LN i | L LN i | L LN i | L LN i
Sheep ID 6 | 7 | 17 | 18 | 8
Histology + | + | - | - | +
-ve +ve
C
0 1 2 3 4 5 6 7 8 9 10 11 12 13
bp
1353
603
310
194
Tissue L LN i | L LN i | LN i | L LN i
Sheep ID 13 | 14 | 15 | 16
Histology - | - | - | -
-ve +ve
D
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
bp
1353
603
310
194
Tissue L LN i | L LN i | L LN i | LN i | L LN i | L LN i
Sheep ID 9 | 19 | 10 | 11 | 20 | 12
Histology + | - | + | + | - | +
-ve +ve
E
A1 A2 A3 A5 A7 A9 A10
A11 A12 A13 A14 B1 B3 B5 B6
B13 B14 B15
D1 D2 D3 D6 D8 D9 D10
D11 D13 D15 D16 D17 -ve +ve

**Table 3.1. Results of single PCR tests on 4, 8 and 12 mg samples of liver, ileocaecal lymph node and ileum, and on 0.5 and 1.0 ml blood samples from 20 sheep suspected of having clinical paratuberculosis and 10 clinically normal control sheep, compared with results obtained by histology and serology.**

| Category | Sheep ID | Histological evidence of paratuberculosis | PCR results | | | | | | | | | | | Serology results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | liver | | | ileocaecal lymph node | | | ileum | | | blood | | AGID | CFT | CSL ELISA | CAHL ELISA |
| | | | 4 mg | 8 mg | 12 mg | 4 mg | 8 mg | 12 mg | 4 mg | 8 mg | 12 mg | 0.5 ml | 1.0 ml | | | | |
| A | 1 | + | + | - | + | nd | nd | nd | + | + | + | + | + | + | +/- | + | + |
| A | 2 | + | + | + | + | - | - | - | + | + | + | + | + | - | - | - | - |
| A | 3 | + | - | + | + | + | + | + | - | + | + | +/- | + | - | - | + | + |
| A | 4 | + | + | - | + | +/- | + | + | + | + | + | + | - | + | - | + | + |
| A | 5 | + | + | - | - | + | - | - | + | + | + | + | - | + | + | + | + |
| A | 6 | + | + | + | + | - | - | - | + | + | + | + | - | + | + | + | + |
| A | 7 | + | - | - | - | + | - | + | + | + | + | + | + | +/- | - | - | - |
| A | 8 | + | + | + | + | + | - | - | + | + | + | - | - | + | + | + | - |
| A | 9 | + | + | + | + | + | + | + | + | + | + | + | +/- | + | + | + | + |
| A | 10 | + | - | + | - | +/- | - | - | + | + | + | - | +/- | +/- | +/- | + | + |
| A | 11 | + | nd | nd | nd | + | + | + | + | + | + | + | - | + | - | + | + |
| A | 12 | + | + | + | - | + | + | + | + | + | + | - | - | + | +/- | + | + |
| A | 13 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| A | 14 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A | 15 | - | - | - | - | nd | nd | nd | - | - | - | - | - | - | - | - | - |
| A | 16 | - | - | - | - | - | - | - | - | - | - | - | - | nd | nd | nd | nd |
| A | 17 | - | - | - | - | - | - | - | - | - | +/- | + | - | - | - | - | - |
| A | 18 | - | - | - | - | - | - | + | - | - | - | - | + | - | - | - | - |
| A | 19 | - | - | - | - | - | - | - | +/- | - | - | + | + | - | - | - | - |
| A | 20 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| B | 21 | - | - | - | - | - | - | - | - | - | - | - | - | +/- | - | - | - |
| B | 22 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 23 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 24 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 25 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 26 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 27 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 28 | - | nd | nd | nd | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 29 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B | 30 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

**A** sheep suspected of having clinical paratuberculosis
**B** clinically normal control sheep
**+** presence of 400-bp band on the gel and a signal present on the dot blot / granulomatous lesions with AFO's in the ileum and/or ileocaecal lymph node and liver
**+/-** signal present on the dot blot but no visible 400-bp band on the gel
**-** absence of 400-bp band on the gel and no signal on the dot blot / neither granulomatous lesions nor AFO's detected in the ileum, ileocaecal lymph node and liver
**nd** not done

**Figure 3.2.** Results of the PCR on selected samples of blood DNA, an equivalent of approximately 0.5 ml blood, from sheep suspected of having clinical paratuberculosis. Separation of 400-bp PCR products, generated by primers 90 and 91, on 2% gel (A). Dot blot results following hybridisation of the 400-bp PCR products with 194-bp radioactively-labelled probe (B). Dots are labelled with numbers of gel's lanes. Samples in lanes 1, 2, 3, 4, and 5 were classified as positive. Bands of 400-bp PCR products were present in these lanes and results of dot blot analysis were concordant. A sample in lane 6 was classified as suspicious, as no band of 400-bp PCR products was visible on the gel but a weak hybridisation signal was obtained in the corresponding dot.

**Histology**:

\+ presence of lesions consistent with paratuberculosis in the ileum,

\- absence of lesions consistent with paratuberculosis in the ileum.

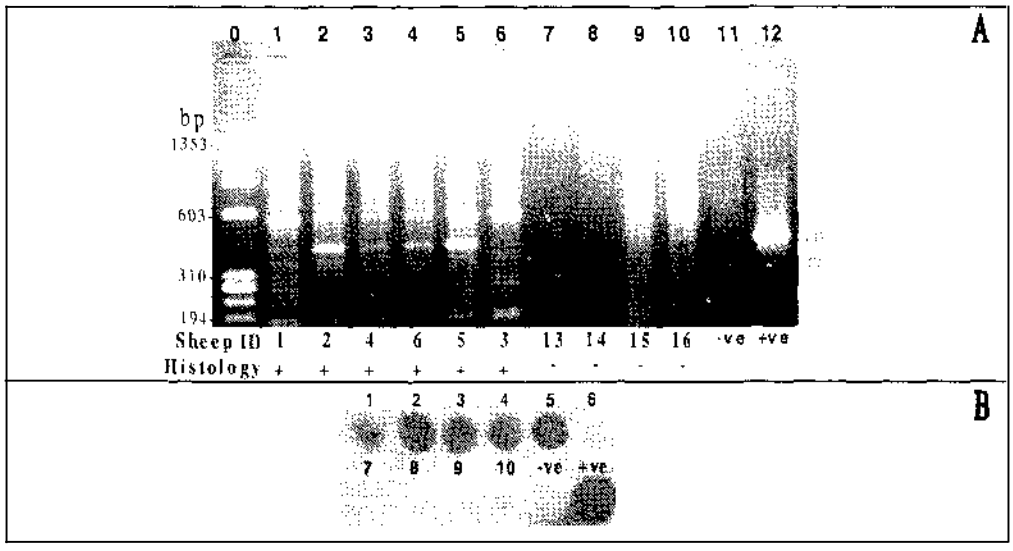
**PCR**:

**-ve** negative control (no DNA)

**+ve** positive control (*M. paratuberculosis* DNA)

**0** molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

**Figure 3.2.**




### 3.3.3. Serology

The results of serological tests are presented in Table 3.1. The sensitivities of the tests, using histology as the standard, were 33% for the CFT, 66% for the AGID, 75% for the CAHL ELISA, and 83% for the CSL ELISA. The combined sensitivity of the tests was 83%.

Of all tests, only CAHL ELISA gave a positive result in one emaciated sheep that had no histological evidence of paratuberculosis and was PCR negative.

All 10 clinically normal control sheep were negative when tested by the CFT, CAHL ELISA and CSL ELISA. The AGID test gave 9 negative results and 1 suspicious reaction in samples from the ten control animals. If this suspicious reaction is ignored the specificity of all serological tests is 100%.

## 3.4. Discussion

The isolation of *M. paratuberculosis* from various extra-intestinal organs and body fluids of infected animals has been reported by numerous authors (Goudswaard, 1971; Hines *et al.,* 1987; Rohde and Shulaw, 1990; Sweeney *et al.,* 1992a; Koenig *et al.,*

1993; Streeter *et al.,* 1995). However, the diagnostic potential of culturing the organism from specimens other than ileocaecal lymph node has been considered to be insignificant (Koenig *et al.,* 1993). Since the PCR is able to detect both viable and nonviable bacteria, an increase in sensitivity would be expected with this method. The application of the PCR to samples of blood for diagnosing of mycobacterial infections in human patients has recently been given credibility (Iralu *et al.,* 1993; Schluger *et al.,* 1994; Rolfs *et al.,* 1995). In addition, *M. paratuberculosis* DNA has been identified in the lung tissue from a sheep with paratuberculosis (Bassett *et al.,* 1993).

In the present study, the PCR assay was applied to samples of purified DNA from ovine blood and solid tissues. The detection rate of *M. paratuberculosis* DNA, when results of single tests were interpreted in duplicate, was 72% for ileocaecal lymph node, 90% for liver, and 100% for ileum in sheep with confirmed paratuberculosis. A single PCR test detected the target DNA in 66% of blood samples using volume of 0.5 ml. Six of 85 samples from 8 clinically affected animals, that had no histological evidence of paratuberculosis, gave positive reactions in the PCR. The interpretation of these inconsistent results is difficult. Although it is believed that most animals which become infected do so shortly after birth (Chiodini *et al.,* 1984b), the ability of the organism to infect adult animals has been reported (Doyle, 1953; Rankin, 1962; Larsen *et al.,* 1975b). On the other hand, even some animals experimentally inoculated with *M. paratuberculosis* do not develop the disease (Brotherston *et al.,* 1961a; Kluge *et al.,* 1968). Thus, the non-concordant histology and PCR results may indicate the detection of early infection in animals that did not have time to develop the typical granulomatous lesions of paratuberculosis. Alternatively, these results may be due to the presence of *M. paratuberculosis* DNA in sheep that had recovered from the infection and no longer had discernible histological lesions. In addition, the possibility of cross-contamination of samples cannot be ruled out. Recognising the limitations of histology, and the uncertain infection status of the 8 clinically affected sheep, the specificity of the assay was assessed on samples from 10 clinically normal animals from a flock where paratuberculosis had not previously been diagnosed. None of the control sheep had histological evidence of infection and all 107 samples from these animals were negative when tested by the PCR, giving the assay 100% specificity.

The isolation of *M. paratuberculosis* from extra-intestinal locations indicates a sporadic

bacteraemia resulting from either direct invasion of blood vessels by the bacilli or access to the circulation through the draining lymphatics (Hines *et al.*, 1987). It is known that macrophages migrate from the intestines to draining lymph nodes (Auger and Ross, 1992). Since mycobacteria are obligatory intracellular parasites of macrophages (Collins, 1988), it is conceivable that the lymphatic route is the main mode of dissemination of the infection.

The most unexpected finding in this study was the low sensitivity of the lymph node PCR. Loss of PCR sensitivity due to the presence of background DNA in clinical samples has previously been reported (Lo *et al.*, 1989). Since lymphocytes constitute the major cell population of the lymph nodes and have a nuclear-cytoplasmic ratio higher than most other cells, the abundance of mammalian DNA may lead to inhibition of the amplification process. Besides the detrimental effect of the vast excess of non-target DNA, the effectiveness of the method used for DNA extraction from *M. paratuberculosis* may not be the same with different tissues. This could provide another explanation for the low detection rate of the lymph node PCR. In addition, the steps employed to purify the DNA could be less successful in removing potential PCR inhibitors from the lymph node samples than the ileum and liver specimens. The possibility that many of the mycobacteria in the hostile lymph node environment were nonviable, and the prokaryotic DNA was already degraded and unsuitable for PCR, cannot be ruled out, however, this seems unlikely. The low sensitivity most likely results from the inadequacy of the method of DNA extraction, along with incomplete sample purification and a quenching effect of mammalian DNA.

The results of the present study illustrate the limitations of serology in the diagnosis of paratuberculosis. All four serological tests, including the AGID test, which gave one unexplained suspicious reaction in one of 10 samples from the clinically normal control animals, were highly (100%) specific. However, none of the tests identified all clinically affected paratuberculous animals and the combined sensitivity of all serological tests was only 83%.

In summary, the present study has demonstrated that *M. paratuberculosis* DNA can be detected in the blood and liver from sheep with clinical paratuberculosis. However, with such a small number of animals tested, it is difficult to comment definitively on the

diagnostic performance of the test. In order to facilitate evaluation of the PCR assay on samples of tissues from subclinically infected sheep, an experimental model of paratuberculosis was developed. This is described in the next chapter. Further assessment of the PCR assay on blood samples and liver biopsy specimens from subclinical sheep, which were infected experimentally with *M. paratuberculosis*, is presented in Chapter 6.

# Chapter 4

# Development of experimental model of paratuberculosis in sheep

## 4.1. Introduction

Paratuberculosis research is hindered by the chronic nature of the disease and low incidence of clinical cases. Experimental models of paratuberculosis provide an opportunity to investigate various aspects of the infection. Among the natural hosts, sheep are frequently chosen as experimental animals because of their relatively low cost (Brotherston *et al.,* 1961a; Brotherston *et al.,* 1961b; Nisbet *et al.,* 1962; Gilmour and Brotherston, 1966; Gilmour *et al.,* 1965; Kluge *et al.,* 1968; Merkal *et al.,* 1968a; Gilmour *et al.,* 1977; Juste *et al.,* 1994). In two of these studies, sheep were infected with *Mycobacterium paratuberculosis* obtained directly from the intestinal mucosa of paratuberculous cattle (Kluge *et al.,* 1968; Merkal *et al.,* 1968a), but in each of the others, isolates grown *in vitro* were used in the inoculum. In one study, sheep were infected with ovine isolates (Gilmour *et al.,* 1977). In the remaining experimental models either bovine isolates alone, or a combination of bovine and ovine isolates, were used for oral inoculation.

Since there is growing evidence that strains which infect sheep differ genetically from strains isolated from cattle (Collins *et al.,* 1990a; de Lisle *et al.,* 1992; Thoresen and Olsaker, 1994; Bauerfeind *et al.,* 1996), data derived from models in which bovine isolates were used to infect sheep may have limited relevance to natural infection. In addition, variation of strain pathogenicity in different host species has been reported (Saxegaard, 1990). It is also possible that organisms grown *in vitro* could have adapted to artificial media and lost their virulence. For example, in one study using a combination of both bovine and ovine isolates in the inoculum, the incidence of intestinal infections was inexplicably low (Gilmour *et al.,* 1965).

The objective of the present study was to develop an experimental model of paratuberculosis in sheep that closely mimics the natural infection by using *M. paratuberculosis* of ovine origin that had never been grown on artificial media.

## 4.2. Materials and methods

### 4.2.1. Preparation of inoculum

The ileum and approximately one meter of the distal jejunum, along with the contents of the caecum, were collected during necropsy from 3 sheep with histologically confirmed paratuberculosis. The intestinal specimens were cut into 10-20 cm long segments and aliquots of approximately 200 g of tissue were transferred to stomacher bags (Seward, UK) containing 200-400 ml phosphate buffered saline [PBS; 0.14 M NaCl, 8 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$; pH 7.2]. The tissue samples were homogenised in a stomacher for 20-30 min, then mixed with the contents of large bowel. In order to remove large particles, the mixture was filtered through gauze. Triplicate aliquots of 1 µl of $10^{-5}$ dilution of the filtrate were stained by Kinyuon method (Hendrickson and Krenz, 1991). Acid-fast organisms (AFO's) in each 1 µl triplicate were counted microscopically (40 x objective). The counts were then averaged and 1 ml of inoculum was estimated to contain $5.2x10^8$ (95%CI: $2.2x10^8$-$8.1x10^8$) organisms. Due to the difficulty in accurately counting mycobacteria, because of clumping, the number of organisms in the inoculum must be regarded as an approximate estimate. The filtrate was stored at 4° C for approximately 1 month and agitated immediately prior to use.

An organism isolated from samples of the inoculum was identified as *M. paratuberculosis* from its slow growth on Herrold's egg yolk medium containing mycobactin, inability to grow on Lowenstein Jensen medium not containing mycobactin, colony morphology and acid-fast staining. The isolation and identification of the organism was performed at The AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand.

### 4.2.2. Animals and husbandry conditions

Thirty new-born lambs, and their dams, were obtained from a flock where paratuberculosis had not previously been diagnosed. The lambs were dosed twice, through a stomach tube, with 20 ml and 45 ml of the inoculum at the age of 1 week and 3-4 weeks respectively. The total dose was calculated to contain approximately $3.4x10^9$ *M. paratuberculosis* organisms.

The lambs were weaned at the age of 7-9 weeks, left on pasture throughout the duration of the study and were supplemented with hay in winter. These animals were monitored, twice a week, for clinical signs of ill-health. Between the 19th and 75th week after oral inoculation with *M. paratuberculosis,* 20 of these 30 sheep developed clinical signs compatible with paratuberculosis and either died (3 sheep) or were sacrificed by intravenous injection of pentobarbitone. The experiment was terminated 108 weeks postinoculation when the remaining 10 experimental sheep were killed by intravenous injection of pentobarbitone.

The parasitic burden in the experimentally infected sheep was controlled by oral administration of 200 µg ivermectin/kg body weight (Ivomec, MSD AGVET, New Zealand) at, on average, 6-weekly intervals throughout the first year of the experiment. During the second year of the study all remaining experimentally infected animals were dosed orally with slow-release anthelmintic (0.5 mg albendazole/kg body weight per day) capsules at 3-monthly intervals according to the manufacturer's (Captec, New Zealand) recommendations. Nematode egg counts, performed in The Department of Veterinary Pathology and Public Health, Massey University, Palmerston North, New Zealand, on faeces collected at, on average, 6-weekly intervals on faecal samples from the experimentally infected animals, were used to monitor the effectiveness of parasite control.

Twelve adult sheep with naturally occurring paratuberculosis (confirmed by PCR), as described in Chapter 3 (3.3.2), were used to compare the lesions produced by natural infection with those produced by experimental infection with *M. paratuberculosis.*

### 4.2.3. Necropsy and sample collection for histology

Necropsy examinations were carried out on 29 experimentally infected sheep. One clinically affected sheep was not available for post-mortem examination. During necropsy, the gross lesions observed were assessed and scored subjectively on a scale from 0 (normal) to 3 (severe). The following samples were collected for histological examination: distal ileum (1 sample of 5 cm, taken 1 cm from the ileocaecal valve), jejunum (1 sample of 5 cm, taken at the level of the caudal mesenteric lymph node) in addition to ileocaecal lymph node, caudal mesenteric lymph node, liver and kidney.

Specimens of other tissues were collected only if lesions were observed during necropsy. For comparative purposes, similar samples were collected from 12 adult sheep with naturally occurring paratuberculosis, as described in Chapter 3.

### 4.2.4. Collection and processing of samples for serology and PCR

***Blood for serological testing***

Samples of blood were collected from the jugular vein of each sheep into 4 ml plain evacuated tubes, initially 10 weeks postinoculation and then 19, 23, 27, 36, 40, 45, 49, 53, 64, 74, 82, 91, 100 and 108 weeks after inoculation. After separation from the clot, samples of serum were initially frozen at -20°C for 2-7 days and then stored -70°C for subsequent serological analysis

***Tissue samples for PCR***

Immediately after euthanasia, and prior to necropsy, samples of ileum and ileocaecal lymph node were collected for PCR from each experimental sheep using clean, sterile instruments and containers. The samples were weighed, transferred in a sterile manner to stomacher bags and DNA was extracted from these samples using the Proteinase K digestion-CTAB-phenol:chloroform extraction method described in Chapter 2 (2.2.2.2). Each sample of extracted DNA was eluted in 120 µl of sterile, distilled water and 10 µl (equivalent of approximately 4 mg of tissue) was tested by the PCR.

### 4.2.5. Polymerase Chain Reaction

***Amplification***

Duplicate samples of DNA extracted from the ileum and ileocaecal lymph node were tested by the PCR using the Expand Long Template system (Boehringer Mannheim, Germany) under standard conditions described in Chapter 2 (2.2.3). Each DNA sample was amplified in 50 µl of PCR mixture containing 2 µM of each primer (90 and 91), 50 mM of Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.5 mM of each dNTP, and 2.625 units of Taq/Pwo polymerase mixture, covered by mineral oil. The reactions were performed using sterile procedures and following contamination-free guidelines as described in Chapter 2 (2.2.3). For each set of reactions, positive (*M. paratuberculosis* DNA) and negative (water and reagents) controls were included.

***Analysis of PCR products***

The analysis of PCR products consisted of electrophoresis, followed by visual examination of 10 µl aliquots of PCR products in 2% agarose gel as described in Chapter 2 (2.2.6). The identity of 400 base pair (bp) amplicons (products of the 90 and 91 primers) was confirmed by dot blot hybridisation with a 194-bp probe that was generated with primers JG1 and JG2 that bind internal to the amplification product of primers 90 and 91. All primers were manufactured by GIBCO BRL, New Zealand.

In order to facilitate the analysis of a large number of samples and to avoid handling of hazardous radioactive material, the probe was labelled with digoxigenin (DIG) using the DIG DNA labelling and detection system (Boehringer Mannheim, Germany). The protocol of the probe labelling was as follows: approximately 2 ng of *M. paratuberculosis* DNA was amplified in the PCR reaction mixture containing 2 µM of each primer (JG1 and JG2), 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dATP, dCTP and dGTP, 0.13 mM dTTP, 0.07 mM DIG-11-dUTP and 5 units of Taq polymerase in a total volume of 100 µl covered by mineral oil (Sigma, USA). The cycling conditions with the Perkin-Elmer Gene Amp 9600 were 1 cycle at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min with a 5 sec extension per cycle. A negative control (water and reagents) was included. The estimation of the yield of the DIG-labelled probe was performed by comparing intensity of signals from serial dilutions of the DIG-labelled probe against serial dilutions (starting from 1 ng/µl to 0.01 pg/µl) of DIG-labelled control DNA (Boehringer Mannheim, Germany).

For dot blot hybridisation, amplification mixtures in each PCR tube were heated at 94°C for 4 min, immediately transferred on ice, and then 1 µl aliquots of each reaction mixture deposited manually onto a Hybond-N membrane (Amersham, UK). After DNA cross-linking by ultraviolet light irradiation for 3.5 min, the membrane was hybridised at 65°C for 2 h in 7-10ml of Rapid-hyb buffer (Amersham, UK) containing approximately 5 ng of the DIG-labelled 194-bp probe per 1 ml of the buffer. The blot was then washed under stringent conditions, as described in Chapter 2 (2.2.6), and the detection of PCR products was carried out using the DIG DNA labelling and detection system according to the manufacturer's recommendations (Boehringer Mannheim,

Germany) (see Appendix A.1 for details).

***Interpretation of PCR results***

The results were interpreted using the interpretation scheme described in Chapter 3 (3.2.4) with one modification. The reaction was classified as positive if, in any of the duplicate samples, a band of the right size (400-bp) was identified on a gel, the hybridisation results were concordant, and no signal was obtained with the negative control. The reaction was considered as suspicious if any of the duplicate samples gave a hybridisation signal on a dot blot but no 400-bp band was present in the corresponding lane.

## 4.2.6. Histology

After fixation in 10% buffered formol saline, tissues were routinely processed, embedded in paraffin and sectioned at 4 μm. Two sections of each tissue sample were prepared. One of the sections was stained with hematoxylin and eosin and the second by Ziehl-Neelsen's method.

Aggregates of 10 or more macrophages were classified as granulomas. Histological lesions observed were assessed and scored subjectively on a scale from 0 (normal) to 3 (severe, diffuse granulomatous infiltration or high number of AFO's).

## 4.2.7. Serological testing

The complement fixation test (CFT) and the gel diffusion test (AGID) were performed as described previously (Hilbink *et al.,* 1994) at The Central Animal Health Laboratory, Wallaceville, Upper Hutt, New Zealand. The enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's (CSL, Australia) recommendations (see Appendix A.2 for details). Serum samples collected sequentially from an individual animal were tested in duplicates on the same plate. The results of each plate were considered valid if the mean optical density (OD), read at 450 nm, of the negative controls was <0.15, the mean OD of the positive controls was between 0.9 and 1.2, and values of the positive controls had less than 30% deviation from their mean OD. Samples were re-tested when the coefficient of variation of their duplicates OD exceeded 30%. The cut-off value of 0.1 (recommended by the manufacturer) plus the

mean of negative controls, was used to interpret the results. Test samples with mean OD values equal to or greater than the predetermined cut-off value were considered positive.

In addition, to facilitate the analysis of the kinetics of the antibody response, the optical density results were converted to antibody indices by dividing the mean OD of each serum sample by the mean OD of the plate positive control.

### 4.2.8. Microscopic examination of faeces for AFO's

Samples of faeces were collected from the rectum of experimentally infected animals at, on average, 6-weekly intervals. Faecal smears were prepared, stained by the Kinyuon method and examined microscopically (40 x objective) for the presence of AFO's. Faecal samples from one month-old lambs obtained from a flock where paratuberculosis had not previously been diagnosed, were used as negative controls.

Results were considered positive when AFO's were present in clumps and their morphology closely resembled the appearance of AFO's in a positive control sample from a sheep with histologically confirmed paratuberculosis. Samples with singular, non-clumped acid-fast bodies were classified as suspicious.

### 4.2.9. Statistical analysis

The ELISA antibody indices in experimental sheep (presented in Figure 4.10) were analysed by non-parametric Kruskal-Wallis/Dunn's multiple comparison test (GraphPad Prism, version 2.01, GraphPad Software Incorporated, USA).

## 4.3. Results

### 4.3.1. Clinical signs and results of microscopic examination of faeces for AFO's

As presented in Figure 4.1, 20 of 30 experimentally infected sheep developed clinical signs compatible with paratuberculosis between the 19th and 75th week after oral inoculation with *M. paratuberculosis*. Ten of the 20 clinical cases occurred between the

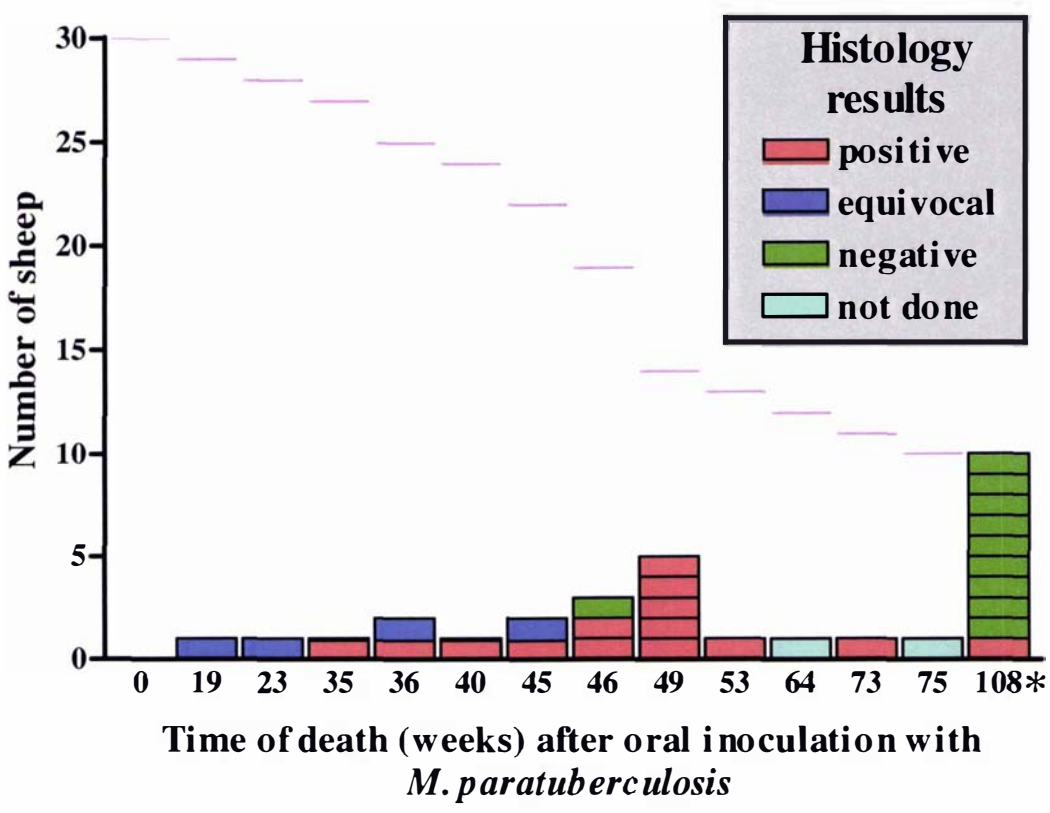
**Figure 4.1.** Death rate of 30 sheep infected orally with *M. paratuberculosis* as lambs. The death rate is indicated by bars. The time of death (indicated by box) of individual sheep is illustrated in relation to histology results. The asterix indicates the time of termination of the study, 108 weeks postinfection.

**Histology results:**

Positive granulomatous lesions with AFO's in the ileum
Equivocal granulomatous lesions without AFO's in the ileum
Negative neither granulomatous lesion nor AFO's detected in the ileum

45th and 53rd week after inoculation, during late winter and early spring. The remaining 10 sheep were clinically normal throughout the duration of the study (108 weeks postinoculation). The earliest clinical signs were exercise intolerance and depression progressing over 3-4 weeks to lethargy. These changes were accompanied by a gradual loss of condition. Diarrhoea was noticed in one animal prior to death. Loss of appetite was observed in 2 moribund sheep.

**Figure 4.1.**




As presented in Table 4.1, clumps of acid-fast organisms were detected in faeces from 9 sheep that developed clinical signs of progressive weakness. In 7 of these 9 animals, the time of the detection of AFO's in faecal smears coincided with the development of clinical signs compatible with paratuberculosis. In the remaining 2 sheep, AFO's were detected prior to the onset of clinical signs suggestive of paratuberculosis.

**Table 4.1. Results of microscopic examination of faeces for acid-fast organisms in 30 sheep infected experimentally with *M. paratuberculosis* compared with histology results.**

| Category of sheep | Sheep ID | Detection of AFO's in faeces: Time after inoculation / weeks | | | | | | | | | | | | | | | Histology results* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 19 | 23 | 27 | 31 | 36 | 40 | 45 | 49 | 53 | 64 | 74 | 82 | 91 | 100 | 108 | |
| Clinically normal sheep | 2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 8 | - | +/- | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 18 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 22 | - | - | +/- | - | - | - | +/- | - | - | - | - | - | - | - | - | - | - |
| | 25 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 26 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 28 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 7 | - | nd | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| Sheep that showed progressive loss of condition | 1 | - | - | - | - | - | - | - | - | +/- | | | | | | | | + |
| | 4 | - | - | - | - | - | - | - | nd | + | | | | | | | | + |
| | 5 | - | - | - | - | - | - | - | - | - | | | | | | | | + |
| | 6 | - | - | - | - | - | - | - | - | - | - | - | + | | | | | + |
| | 11 | - | - | nd | - | - | - | | | | | | | | | | | + |
| | 13 | - | nd | - | +/- | - | + | | | | | | | | | | | + |
| | 17 | nd | - | - | +/- | - | - | - | + | | | | | | | | | + |
| | 19 | - | - | - | - | - | - | - | - | | | | | | | | | + |
| | 20 | - | nd | - | - | - | - | - | | | | | | | | | | + |
| | 23 | - | - | nd | - | - | - | - | + | | | | | | | | | + |
| | 24 | - | - | - | - | - | - | - | - | - | | | | | | | | + |
| | 27 | - | - | - | - | - | - | - | + | - | + | | | | | | | + |
| | 29 | - | - | - | nd | - | +/- | - | - | + | | | | | | | | + |
| | 9 | nd | - | - | - | - | - | | | | | | | | | | | +/- |
| | 12 | - | - | - | +/- | - | - | - | - | | | | | | | | | - |
| | 14 | - | - | | | | | | | | | | | | | | | +/- |
| | 16 | - | - | - | - | - | - | - | - | | | | | | | | | +/- |
| | 30 | - | - | - | | | | | | | | | | | | | | +/- |
| | 21 | - | - | - | - | - | - | - | - | - | - | + | - | | | | | nd |
| | 10 | - | - | - | - | - | - | - | - | - | + | | | | | | | nd |

* presence or absence of lesions consistent with paratuberculosis in the ileum
\+ clumps of acid fast organisms in faeces or typical lesions (with AFO's)
+/- individual acid fast bodies in faeces or equivocal lesions (without AFO's)
\- absence of acid fast bodies in faeces or lesions not detected
**nd** not done

## 4.3.2. Gross pathology

One of the twenty sheep that developed clinical signs of progressive weakness was not available for necropsy. Gross findings in 29 experimentally infected sheep and 12 sheep with naturally occurring paratuberculosis are summarised in Table 4.2. In the 14 experimental sheep with histologically confirmed paratuberculosis, the enlargement of mesenteric lymph nodes and thickening of serosal lymphatics, draining ileum and distal jejunum, were the most consistent macroscopic findings, while the thickening of the wall of the ileum was noted in 6 cases.

**Table 4.2. Necropsy findings compared with results of histological examination in 29 sheep infected experimentally with *M. paratuberculosis* and in 12 sheep with naturally occurring paratuberculosis.**

| Category of sheep | Histological evidence of paratuberculosis | | Number of sheep with lesions | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Body condition | | | Thickening of ileum | | | | Enlargment of ileocaecal lymph node | | | | Prominence of serosal lymphatics |
| | | | good | light | emaciated | absent | mild | moderate | severe | absent | mild | moderate | severe | |
| Sheep with naturally occurring paratuberculosis | + | n=12 | 1 | 3 | 8 | 1 | 3 | 3 | 5 | 0 | 1 | 6 | 5 | 11 |
| Sheep infected experimentally with *M. paratuberculosis* that showed progressive loss of condition | + | n=13 | 0 | 9 | 4 | 7 | 2 | 1 | 3 | 0 | 0 | 6 | 7 | 11 |
| | +/- | n=5* | 0 | 2 | 3 | 3 | 0 | 1 | 1 | 0 | 0 | 0 | 4 | 4 |
| | - | n=1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| Sheep infected experimentally with *M. paratuberculosis* that were clinically normal throughout the experiment | + | n=1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | - | n=9 | 9 | 0 | 0 | 6 | 1 | 2 | 0 | 5 | 3 | 1 | 0 | 0 |

**Histology** (presence or absence of lesions consistent with paratuberculosis in the ileum):
+ typical granulomatous lesions (with AFO's)
+/- equivocal granulomatous lesions (without AFO's)
- neither granulomatous lesions nor AFO's detected
* Tissue sections from one experimental sheep that developed clinical signs of progressive loss of condition were not available for histology. However, the detection of AFO's in faeces from this animal indicates that it most likely had clinical paratuberculosis.

### 4.3.3. Histology

The results of histological examination of intestinal sections from 28 sheep infected experimentally with *M. paratuberculosis*, and 12 sheep with naturally occurring paratuberculosis, are summarised in Table 4.3. Tissue sections from 2 experimental sheep that developed clinical signs of progressive loss of condition were not available for histology. However, the detection of AFO's in faeces from these 2 animals indicates that they most likely had clinical paratuberculosis.

Fourteen of the 28 experimentally infected sheep examined, one of the 10 clinically normal animals that survived to the end of the experiment, and 13 of the 18 sheep which developed progressive loss of condition, had enteric lesions typical of paratuberculosis. In 9 of the 13 experimentally infected sheep with clinical paratuberculosis, and in all 12 naturally infected sheep, the ileal and jejunal lamina propria were diffusely infiltrated with macrophages. This diffuse infiltration was associated with severe atrophy of villi, characterised by clubbing and fusion of villi, and compression and obliteration of the intestinal crypts. These changes were accompanied by variable infiltration of the lamina propria with lymphocytes, eosinophils and occasional aggregates of neutrophils (Figures 4.2. and 4.3). In the remaining 5 experimentally infected sheep with clinical paratuberculosis, and in the one experimental sheep that survived to the end of the experiment, there were multifocal aggregates of closely packed macrophages, usually located in the tips of intestinal villi (Figure 4.4). Furthermore, in these 6 sheep the ileal and jejunal lamina propria was diffusely infiltrated with lymphocytes and eosinophils, and contained occasional aggregates of neutrophils. These changes were accompanied by mild to moderate shortening and clubbing of some villi, along with obliteration of some of the intestinal crypts. Numerous AFO's were present within macrophages in intestinal sections from all 12 naturally infected sheep and 12 experimentally infected sheep with clinical paratuberculosis . The organisms were sparse in the single remaining experimental sheep with clinical paratuberculosis and in the single clinically normal experimental sheep that survived to the end of the experiment.

**Table 4.3. Results of histological examination of sections of ileum and mesenteric lymph node from 28 sheep infected experimentally with *M. paratuberculosis* and 12 sheep with naturally occurring paratuberculosis.**

| Site | Type of lesions | Number of sheep with lesions | | | |
|---|---|---|---|---|---|
| | | Sheep infected experimentally with *M. paratuberculosis* (n=28) | | Sheep with naturally occurring paratuberculosis (n=12) | |
| Intestinal mucosa | Diffuse granulomatous infiltration | 9 | (9) | 12 | (12) |
| | Focal granulomas | 9 | (5) | - | - |
| Intestinal submucosa | Diffuse granulomatous infiltration | 1 | (1) | 8 | (8) |
| | Focal granulomas | 14 | (13) | 4 | (4) |
| Submucosal nerves | Mononuclear infiltration | 5 | (1) | 8 | (2) |
| Serosal lymphatics** | Intraluminal nests of macrophages | 11 | ** | 12 | ** |
| Mesenteric lymph node | Focal granulomas | 18 | (11) | 12 | (11) |

Figures in brackets indicate the number of sheep with AFO's

** site not examined for AFO's

Of the remaining 5 experimental sheep that developed clinical signs suggestive of paratuberculosis, 4 had equivocal granulomatous lesions without AFO's in the ileal and jejunal mucosa. The lesions consisted of multifocal aggregates of loosely packed macrophages, moderate numbers of lymphocytes and eosinophils, and occasional aggregates of neutrophils in the ileal and jejunal lamina propria. There was mild atrophy of villi with compression and obliteration of some of the intestinal crypts, and in one animal that died 19 weeks after experimental infection with *M. paratuberculosis*, aggregates of cells resembling macrophages were present within Peyer's patches (Figure 4.5).

**Figure 4.2.** Small intestine from a sheep with naturally occurring paratuberculosis. The lamina propria shows diffuse infiltration with macrophages. H & E x 70

**Figure 4.3.** Small intestine from a sheep infected experimentally with *M. paratuberculosis*. The lamina propria shows diffuse infiltration with macrophages. H & E x 90

**Figure 4.2.**

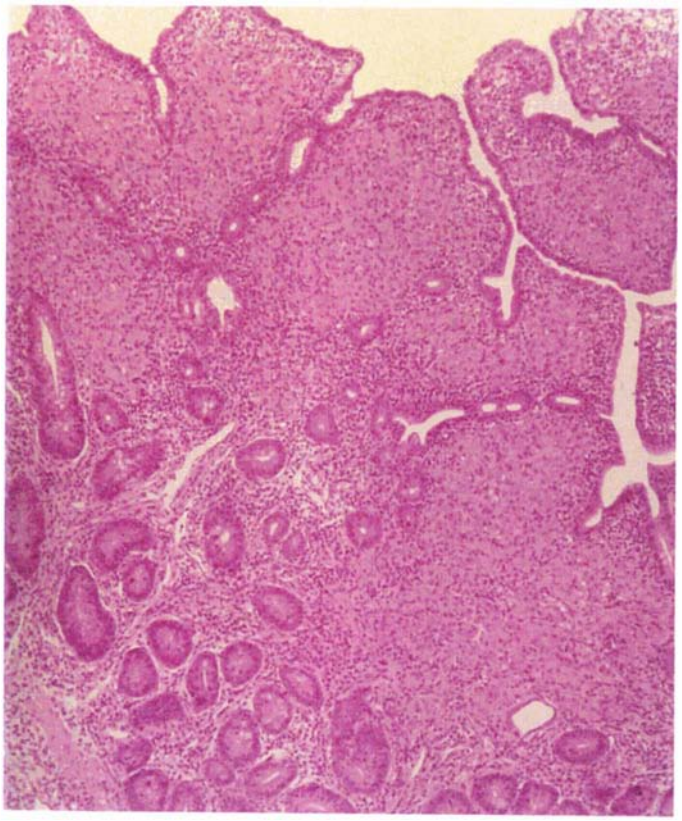


**Figure 4.3.**

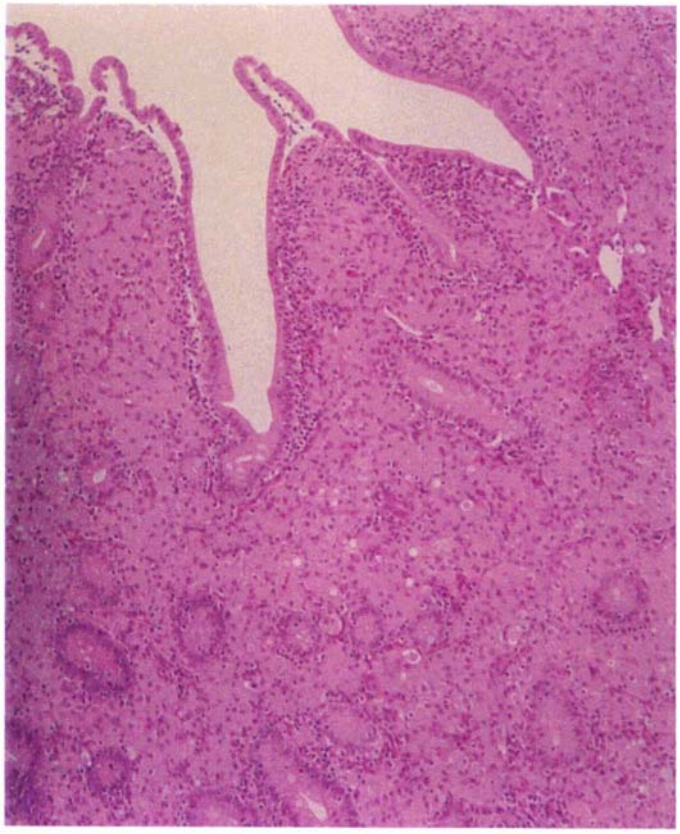


**Figure 4.4.** Small intestine from a sheep infected experimentally with *M. paratuberculosis*. Aggregates of macrophages ↑ in the lamina propria. H & E x 100

**Figure 4.5.** Small intestine from a sheep infected experimentally with *M. paratuberculosis*. Small aggregates of macrophages ↑ in the ileal Payer's patch. H & E x 70

**Figure 4.4.**

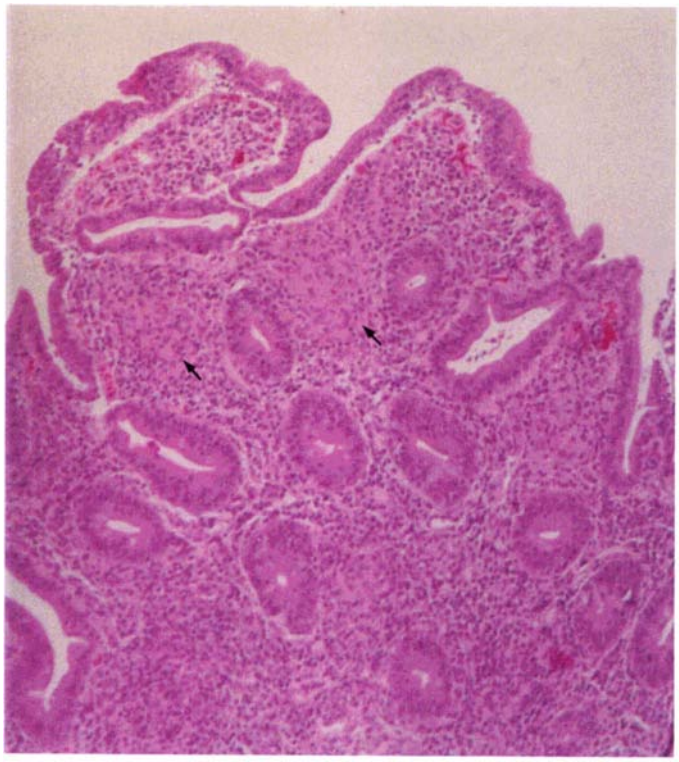
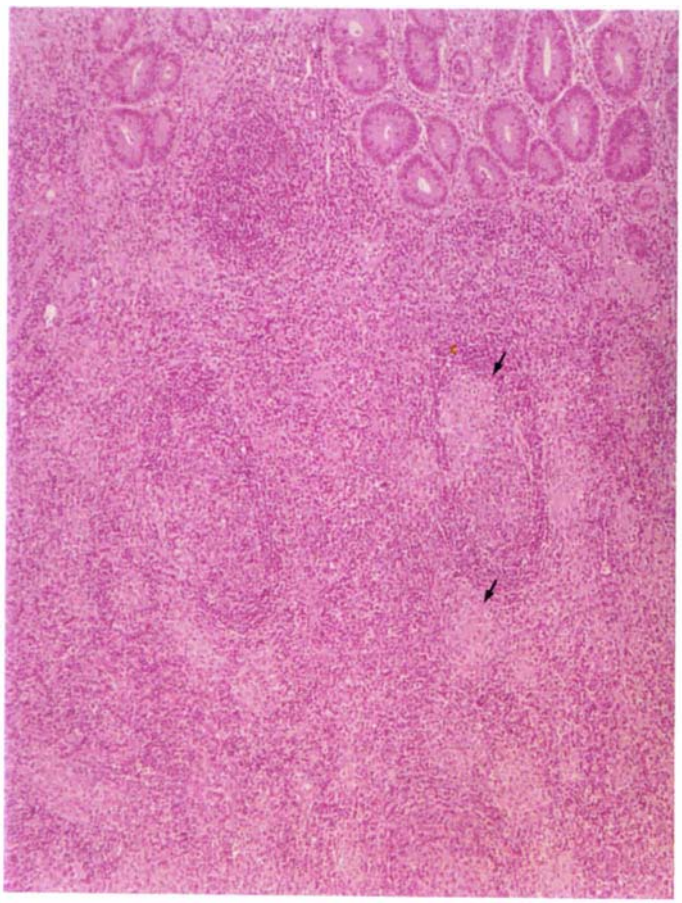


**Figure 4.5.**



In all 12 sheep naturally infected with paratuberculosis, and all 14 experimental sheep with enteric lesions of paratuberculosis, the granulomatous infiltration extended from the intestinal mucosa to the submucosa. This infiltration was most severe in the submucosa adjacent to the mucosa. Focal aggregates of macrophages accompanied by mild lymphoid cell infiltration were also evident in the submucosa in one of the 4 experimental sheep with equivocal enteric lesions (without AFO's). In addition, in 8 of the 12 naturally infected sheep and in 5 of the 14 experimental sheep with enteric lesions of paratuberculosis, there were accumulations of mononuclear cells around some nerves in the submucosa and in the muscular layer (Figures 4.6. and 4.7). In some cases this infiltration of epineural interstitium apparently extended between the nerve fascicles into the perineurium. Most of the cells resembled lymphocytes, although occasional plasma cells and macrophages were also discernible in the infiltrate. In 2 naturally infected sheep, and in one experimental sheep, sparse AFO's were detected within some of the mononuclear cells (Figure 4.7).

In all 12 naturally infected sheep, and in 10 of the 14 experimental sheep with enteric lesions of paratuberculosis, the serosal lymphatics of the ileum and jejunum were surrounded by a zone of mononuclear cells and contained nests of lymphocytes and macrophages. Similar lesions were also present in one of the 4 experimental sheep with equivocal enteric lesions. Multinucleate giant cells were present within the lumen of the affected lymphatics in one naturally infected sheep and in 3 experimental sheep.

Aggregates of macrophages were present in the cortices of ileocaecal lymph nodes and caudal mesenteric lymph nodes of the 12 naturally infected sheep, 14 experimental sheep with enteric lesions of paratuberculosis, and 4 sheep with equivocal enteric lesions. The number and size of these microgranulomas varied between animals. In some cases as few as ten macrophages constituted a microgranuloma, while in other cases the mononuclear cells were too numerous to count. Within the group of naturally infected sheep, AFO's were numerous in mesenteric lymph nodes of 6 sheep, sparse in five, and in a lymph node of one sheep, there was no evidence of organisms. In comparison, in the 14 experimental sheep with enteric lesions of paratuberculosis AFO's were numerous in one mesenteric lymph node, moderate in 2, sparse in 8, and in 3 lymph nodes AFO's were not detected. No AFO's were evident in sections of mesenteric lymph node from the 4 sheep with equivocal enteric lesions.

**Figure 4.6.a.** Small intestine from a sheep with naturally occurring paratuberculosis. Accumulation of mononuclear cells ⇑ around the submucosal nerve, **m**-mucosa, **ml**-muscular layer. H & E x 100

**Figure 4.6.b.** Higher magnification of the accumulation of mononuclear cells ⇑ around the submucosal nerve ↑. H & E x 440

Figure 4.6.a.

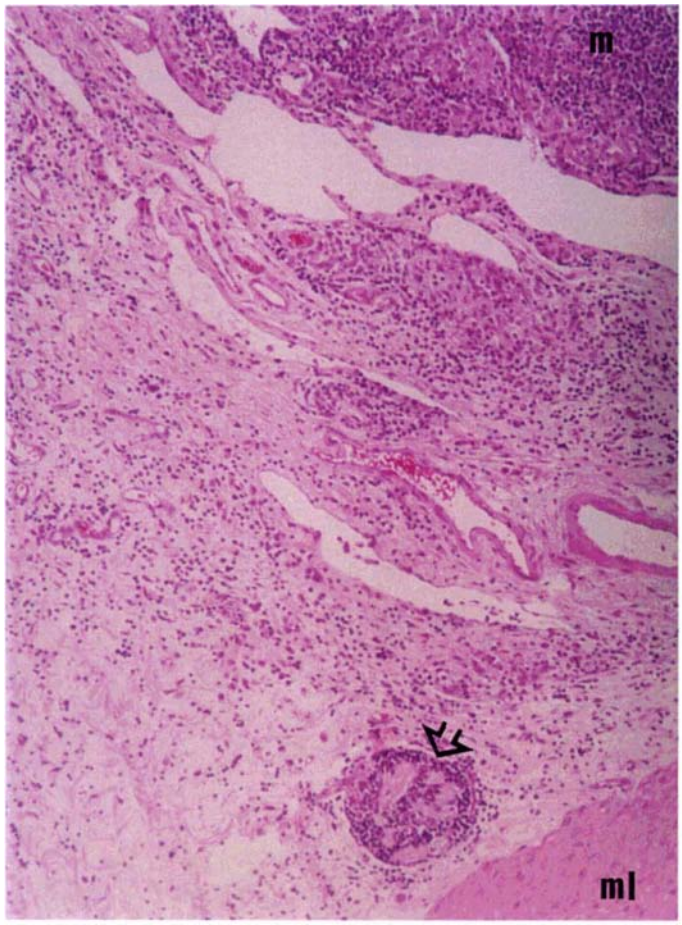



Figure 4.6.b.

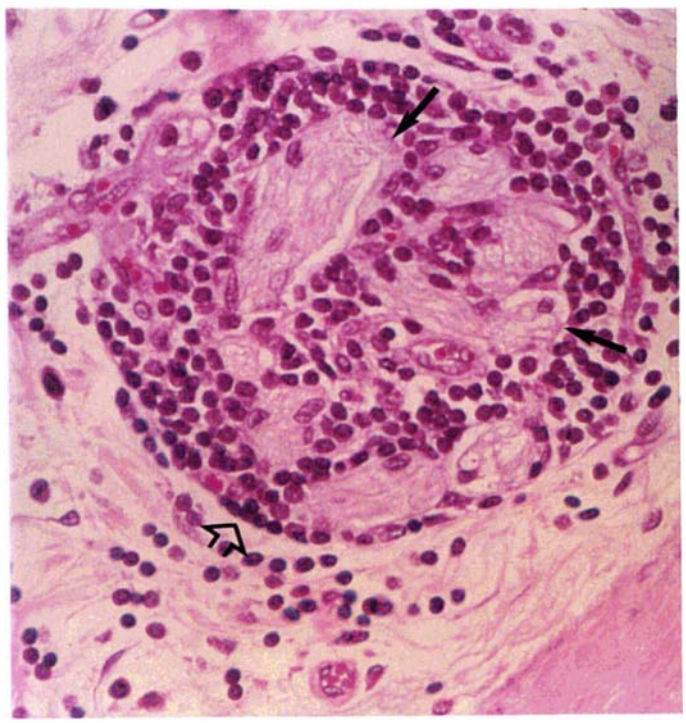


**Figure 4.7.a.** Small intestine from a sheep with naturally occurring paratuberculosis. Accumulation of mononuclear cells ⇑ around a nerve in the muscular layer (**ml**). The submucosa (**s**) shows diffuse infiltration with macrophages. H & E x 100

**Figure 4.7.b.** Higher magnification of the accumulation of mononuclear cells ⇑ around the nerve ↑ in the muscular layer. H & E x 260

**Figure 4.7.c.** Singular acid-fast organisms ↑ within some of the mononuclear cells surrounding the nerve in the muscular layer. Z-N x 540

Figure 4.7.a.

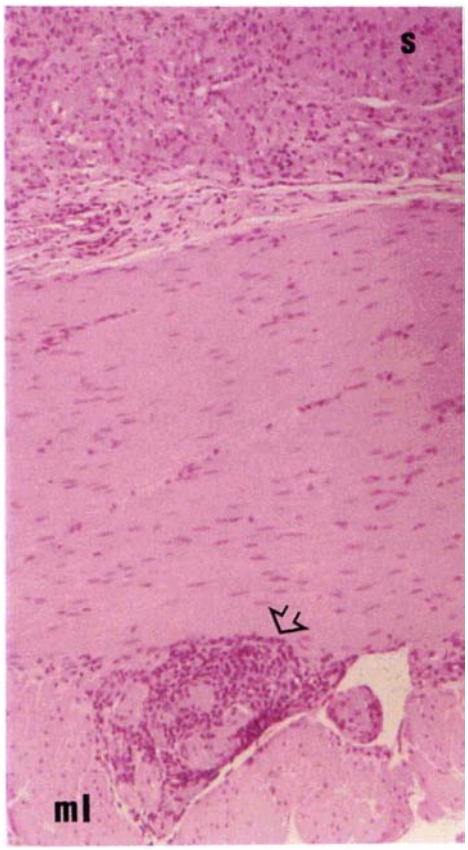



Figure 4.7.b.

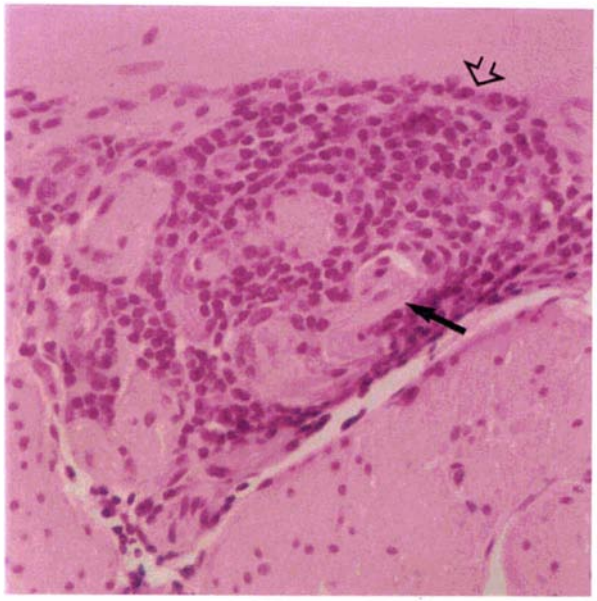
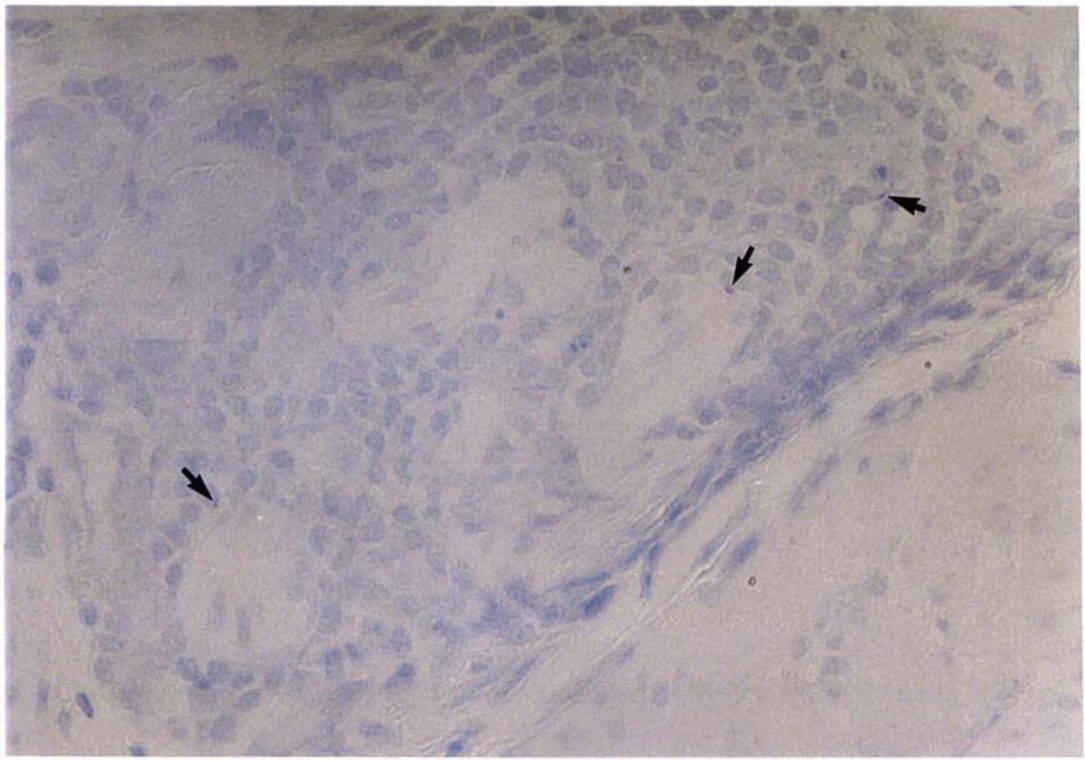


Figure 4.7.c.



One of the 18 experimental sheep, which developed progressive loss of condition, and 9 of the 10 clinically normal sheep that survived to the end of the experiment, had neither granulomatous lesions nor AFO's in sections of intestine and mesenteric lymph node. None of the section of intestine from these 10 sheep showed accumulation of mononuclear cells around nerves in the intestinal submucosa or in the muscular layer.

### 4.3.4. Polymerase Chain Reaction

Results obtained by duplicate PCR tests on samples of ileum and ileocaecal lymph node from sheep experimentally infected with *M. paratuberculosis* are summarised in Table 4.4. Similar numbers of experimental sheep tested positive or suspicious by the PCR on samples of ileum and ileocaecal lymph node were identified as infected by histology. The PCR test gave positive reactions in 13 of 14 ileal samples from 14 sheep with histological evidence of paratuberculosis, 7 of which also tested positive by the PCR on samples of ileocaecal lymph node. In addition, 2 suspicious results were obtained in samples of ileocaecal lymph node from these 14 sheep. On 4 occasions, the PCR gave negative results in tissues from 4 animals with granulomatous lesions where AFO's could be seen microscopically. Three positive reactions and one suspicious result were obtained by the PCR in tissue samples, either ileum or ileocaecal lymph node, from 3 animals with equivocal lesions where AFO's could not be detected microscopically. The PCR was also positive on ileal tissue of one animal from which tissue samples were not available for histology but which had AFO's in its faeces.

None of the tissue samples from 10 experimental sheep without histological evidence of paratuberculosis (9 clinically normal and one sheep which developed progressive loss of condition) was PCR-positive.

The results of the PCR on selected tissues of sheep experimentally infected with *M. paratuberculosis* are presented in Figure 4.8.

**Table 4.4. Results of the PCR on duplicate samples of ileum and ileocaecal lymph node from 30 sheep infected experimentally with *M. paratuberculosis* compared with results obtained by histology and by 3 different serological tests.**

| Category of sheep | Sheep ID | Histology and PCR results | | | | Serological evidence of paratuberculosis (No. of +ve results per No. of samples tested) | | |
|---|---|---|---|---|---|---|---|---|
| | | Ileum | | Mesenteric lymph node | | | | |
| | | Histology | PCR | Histology | PCR | CFT | AGID | ELISA |
| Clinically normal sheep | 2 | - | - | - | - | 0/15 | 0/15 | 0/15 |
| | 3 | - | - | - | - | 1/15 | 0/15 | 0/15 |
| | 8 | - | - | - | - | 2/15 | 1/15 | 0/15 |
| | 15 | - | - | - | - | 11/15 | 9/15 | 13/15 |
| | 18 | - | - | - | - | 0/15 | 9/15 | 1/15 |
| | 22 | - | - | - | - | 0/15 | 5/15 | 0/15 |
| | 25 | - | - | - | - | 3/15 | 3/15 | 4/15 |
| | 26 | - | - | - | - | 6/15 | 12/15 | 1/15 |
| | 28 | - | - | - | - | 2/15 | 1/15 | 0/15 |
| | 7 | + | + | +/- | - | 1/15 | 0/15 | 0/15 |
| Sheep that showed progressive loss of condition | 1 | + | + | + | +/- | 1/8 | 2/8 | 3/8 |
| | 4 | + | - | + | + | 1/8 | 3/8 | 3/8 |
| | 5 | + | + | + | +/- | 2/8 | 1/8 | 0/8 |
| | 6 | + | + | +/- | + | 3/11 | 3/11 | 3/11 |
| | 11 | + | + | + | - | 1/5 | 4/5 | 1/5 |
| | 13 | + | + | + | + | 2/4 | 0/4 | 1/4 |
| | 17 | + | + | +/- | - | 1/7 | 1/7 | 2/7 |
| | 19 | + | + | + | - | 1/7 | 1/7 | 1/7 |
| | 20 | + | + | + | + | 1/6 | 5/6 | 2/6 |
| | 23 | + | + | + | + | 0/7 | 4/7 | 4/7 |
| | 24 | + | + | + | + | 5/8 | 6/8 | 8/8 |
| | 27 | + | + | + | + | 3/9 | 3/9 | 2/9 |
| | 29 | + | + | + | - | 0/8 | 2/8 | 6/8 |
| | 9 | +/- | + | +/- | - | 0/5 | 0/5 | 0/5 |
| | 12 | - | - | - | - | 4/7 | 1/7 | 0/7 |
| | 14 | +/- | nd | +/- | + | 0/2 | 0/2 | 0/2 |
| | 16 | +/- | - | +/- | - | 4/7 | 0/7 | 0/7 |
| | 30 | +/- | + | +/- | +/- | 0/3 | 0/3 | 1/3 |
| | 21 | nd | + | nd | - | 1/11 | 5/11 | 3/11 |
| | 10 | nd | nd | nd | nd | 1/9 | 3/9 | 3/9 |

\+ presence of 400-bp band on the gel and a signal present on the dot blot / granulomatous lesions with AFO's in the ileum

+/- signal present on the dot blot but no visible 400-bp band on the gel / granulomatous lesions without AFO's in the ileum

\- absence of 400-bp band on the gel and no signal on the dot blot / neither granulomatous lesions nor AFO's detected in the ileum

**nd** not done

**Figure 4.8.** Results of the PCR on selected samples of ileal and ileocaecal lymph node DNA from sheep infected experimentally with *M. paratuberculosis* as lambs. Separation of 400-bp PCR products, generated by primers 90 and 91, on 2% gel (A and B). Dot blot results following hybridisation of the 400-bp PCR products with 194-bp DIG-labelled probe (C). Dots are labelled with the alphabetical code of the gel and numbers of gel's lanes. Samples in lanes A1, A2, A5, A8, A11, A12, A13, A14, A18, A19, A20, A21, A22, A23, A25, A26, B3, B4, B9, B10, B19, B20, B24 and B25 were classified as positive. Bands of 400-bp PCR products were present in these lanes and results of dot blot analysis were concordant. Samples in lanes A24, B1, B7 and B8 were classified as suspicious, as no bands of 400-bp PCR products were visible on the gels but hybridisation signals were obtained in the corresponding dots.

**Tissue**:

**i** ileum

**In** ileocaecal lymph node

**Histology** (presence of lesions consistent with paratuberculosis in the ileum):

**+** granulomatous lesions with AFO's

**+/-** granulomatous lesions without AFO's (equivocal lesions)

- neither granulomatous lesions nor AFO's detected

* Tissue sections from one experimental sheep that developed clinical signs of progressive loss of condition were not available for histology.

**PCR**:

-ve negative control (no DNA)

+ve positive control (*M. paratuberculosis* DNA)

**0** molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

**Figure 4.8.**

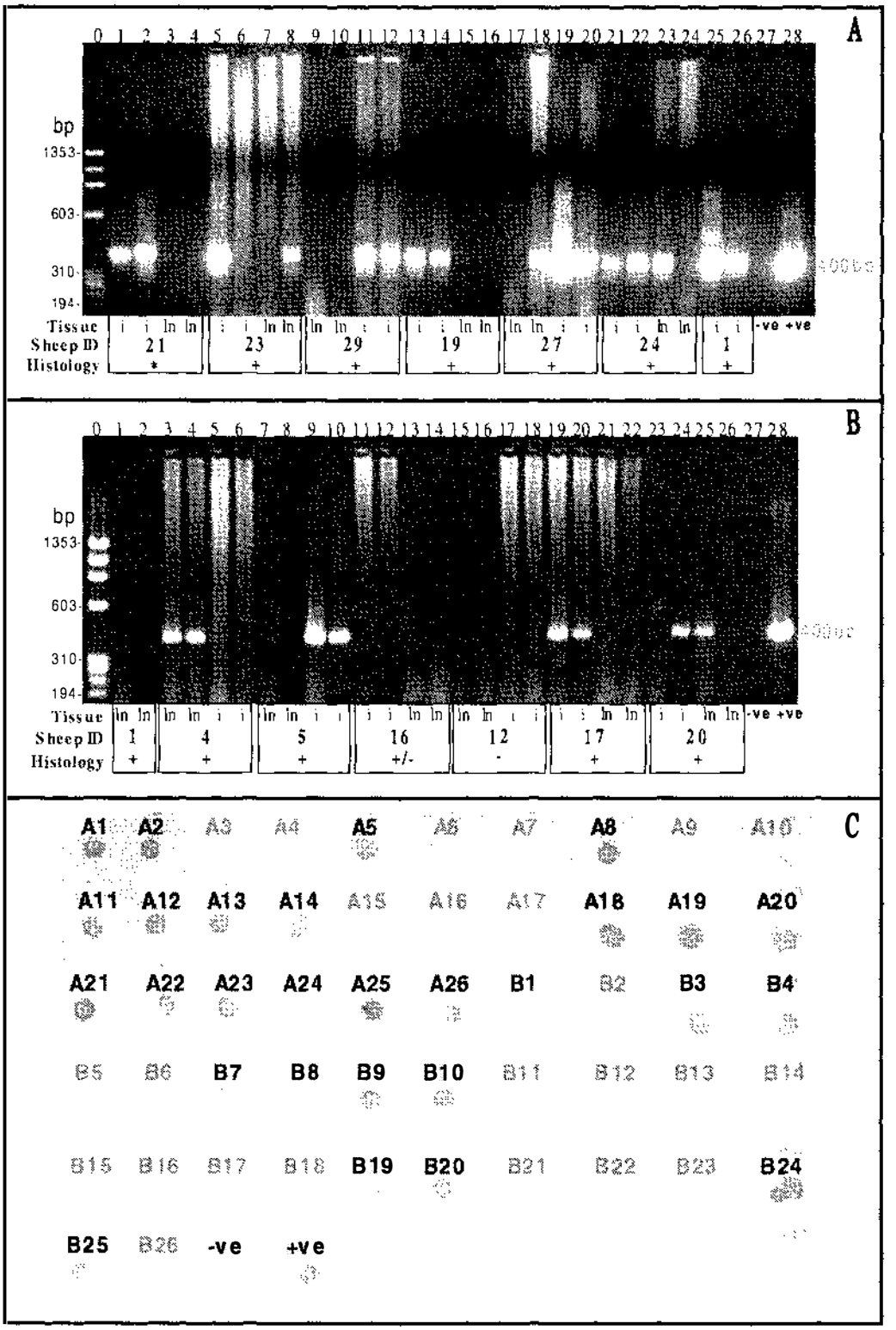
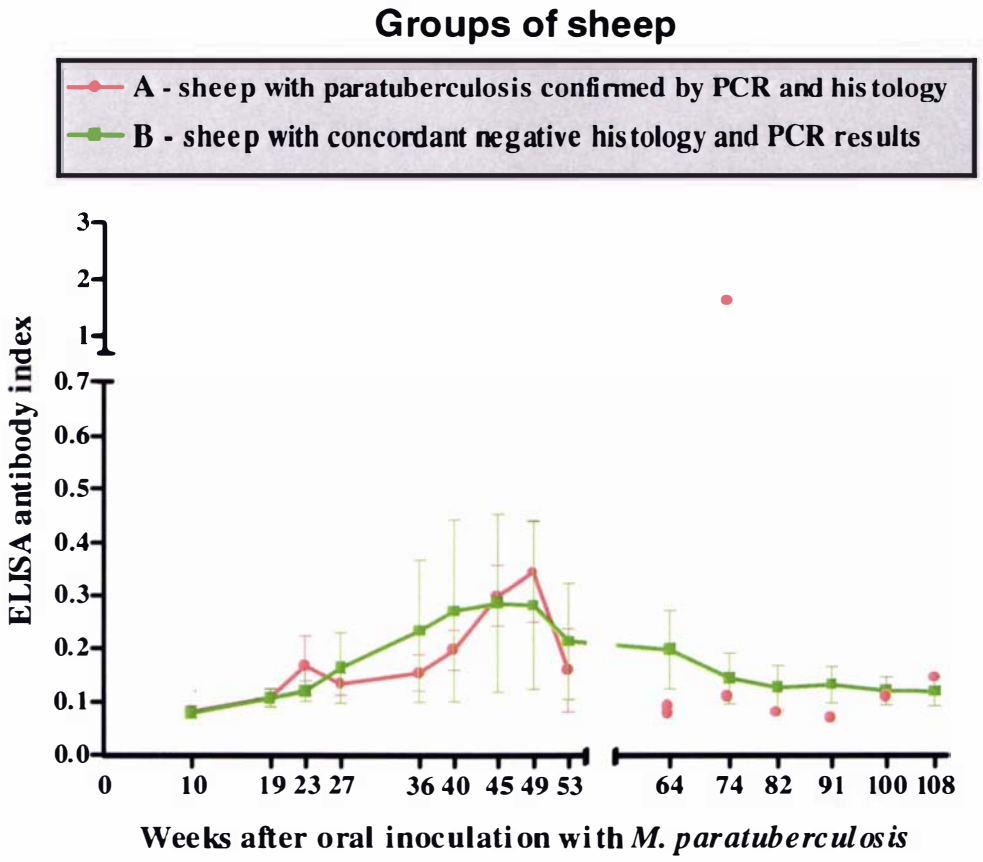

A
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
bp
1353
603
310
194
Tissue
Sheep ID
Histology
21 23 29 19 27 24 1
-ve +ve
B
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
bp
1353
603
310
194
Tissue
Sheep ID
Histology
1 4 5 16 12 17 20
-ve +ve
C
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10
A11 A12 A13 A14 A15 A16 A17 A18 A19 A20
A21 A22 A23 A24 A25 A26 B1 B2 B3 B4
B5 B6 B7 B8 B9 B10 B11 B12 B13 B14
B15 B16 B17 B18 B19 B20 B21 B22 B23 B24
B25 B26 -ve +ve

**Figure 4.9.** ELISA's antibody indices in serum samples of 14 experimental sheep that had infection with *M. paratuberculosis* confirmed by histology and the PCR (Group A) and 9 experimental sheep that were clinically normal throughout the duration of the study (Group B). The 9 clinically normal sheep showed no evidence infection with *M. paratuberculosis*, as determined by the PCR and histology at the time of necropsy, 108 weeks after oral inoculation. Results are presented as mean ±SEM. During the experiment, the number of animals in Group A was declining (see Figure 4.1). The data were analysed by non-parametric Kruskal-Wallis/Dunn's multiple comparison test. Because of the small number of sheep in Group A 53 weeks postinoculation, the analysis was only performed with data obtained between the 10th and 53rd week after inoculation. At all sampling points analysed there was no significant difference ($P > 0.05$) between Groups A and B. Red dots represent antibody indices in each sheep from Group A between the 64 and 108 weeks after inoculation.

### 4.3.4. Serological testing

The results of the AGID, CFT and ELISA in 30 sheep experimentally infected with *M. paratuberculosis* are summarised in Table 4.4. Positive reactions in the serological tests were obtained on more than one occasion in 13 of the 14 sheep in which the infection with *M. paratuberculosis* was confirmed by both histology and PCR (Group A). Among the 9 experimental sheep that survived to the end of the experiment and had concordant negative histology and PCR results (Group B), 7 gave positive reactions on more than one occasion in the serological tests. As presented in Figure 4.9, there was no significant difference (P>0.05) between the median antibody indices in sheep in Group A and Group B. In the 9 experimental sheep that survived to the end of the experiment and showed no evidence of infection with *M. paratuberculosis*, as determined by the PCR and histology, an initial rise in antibody levels between the 10 and 49 weeks postinfection was followed by a declining trend from 49 weeks postinoculation.

**Figure 4.9.**



## 4.4. Discussion

Of the thirty lambs experimentally infected with *M. paratuberculosis*, 20 developed progressive loss of condition and were killed or died within the first 75 weeks of the experiment. The results obtained by histology, PCR and microscopic examination of faeces indicated that 15 of these 20 sheep had clinical paratuberculosis. In addition, infection with *M. paratuberculosis* was confirmed by histology and PCR in one of 10 experimental animals that survived until the end of the experiment, 108 weeks after experimental infection. Lesions detected in sheep experimentally infected with *M. paratuberculosis* were similar to those in 12 naturally infected sheep and closely resembled the lesions described by Carrigan and Seaman (1990), Clarke and Little (1996) and type 3a and 3b lesions reported by Perez et al., (1996). The high incidence of infection, and the similarity between lesions in the experimentally and naturally infected sheep, indicates that the experimental model presented here may be a suitable tool for the study of paratuberculosis.

The age when paratuberculosis is usually diagnosed in naturally infected sheep ranges between 2 and 6 years (Carrigan and Seaman, 1990). However, in endemically infected flocks clinical paratuberculosis has been diagnosed in hoggets younger than 1 year of age (Gumbrell, 1986). In the present study, the incubation time in 13 experimental animals with histologically confirmed clinical paratuberculosis ranged from 35 to 73 weeks after inoculations and their median survival time was 49 weeks. This indicates that the progression of infection in these sheep was faster than in most of natural cases of paratuberculosis, presumably due to the large dose of *M. paratuberculosis* used at an early age.

In New Zealand, most clinical cases of naturally acquired ovine paratuberculosis have been reported to occur in winter (Gumbrell, 1986). It is considered that the stress of pregnancy and the cold weather allows the disease to express itself more readily (Bruère and West, 1993). In the current study, the majority of clinical cases occurred in late winter and early spring, between the 45th and 53rd week of the trial, when environmental conditions were harsh. This coincided with a peak in the antibody response. Antibodies are believed to play little, if any, active role in the resistance to mycobacterial infections, and their production is thought to be stimulated by release of

large amounts of antigen from dead mycobacteria (Collins, 1988). Thus, it is conceivable that stress-induced depression of the immune system (Mims, 1983) permitted the infection to progress to a clinical stage and precipitated shedding of AFO's in faeces of a number of sheep in the present study. Subsequent contamination of pasture might also have led to exogenous re-infection of some animals. Due to limited knowledge of the epidemiology of the agent, it is impossible to determine if the histological lesions of paratuberculosis in one animal that had remained clinically normal until the end of the trial represented a sequel to primary inoculation or re-infection during the course of the experiment.

Of the 20 experimental sheep that developed clinical signs suggestive of paratuberculosis, 4 animals had equivocal histological lesions in the intestines and mesenteric lymph nodes. Although AFO's were not detected in any sections examined from these 4 sheep, the tissue samples from 3 of them gave positive reactions by the PCR. This indicates the presence of the target DNA in the specimens tested. The apparent absence of AFO's in these equivocal lesions may reflect either the low sensitivity of the staining method or the fact that infection had been successfully controlled and only residual lesions, lacking viable organisms, remained. It is possible that organisms in these lesions were in a spheroplast form, or the samples were contaminated. Alternatively, the lesions could have been of parasitic origin following destruction of migrating nematode larvae. In such instance, the positive results obtained by the PCR may just indicate concurrent mild infection with *M. paratuberculosis*.

It has previously been reported that some sheep that had been inoculated experimentally with *M. paratuberculosis* did not develop the disease (Brotherston *et al.,* 1961a; Kluge *et al.,* 1968). In the present study, 9 experimentally infected sheep which survived until the end of the trial were found to have no evidence of infection with *M. paratuberculosis* at the time of necropsy 108 weeks postinfection. Seven of the nine sheep showed serological evidence of infection on more than one occasion. Since the antibody assays used in this study have been reported to be highly specific (Hilbink *et al.,* 1994), it is conceivable, that these 7 animals had been successfully infected but were able to counter the challenge of exposure to *M. paratuberculosis.* This is further supported by the fact that in the group of these 9 experimental sheep, an initial rise of antibody titre was followed by a declining trend, presumably due to a decrease in

antigenic stimulation subsequent to elimination or confinement of the infection. However, the possibility of persistent infection, below the level that could be detected by either the PCR method or histology, cannot be excluded. Because these sheep were sacrificed 2 years after experimental infection, it was impossible to determine if they were at an advantage in the long term. Interestingly, there was no significant difference ($P>0.05$) between median antibody indices in the 14 experimental sheep with histological evidence of paratuberculosis, and in the 9 experimental sheep with concordant negative histology and PCR results. This suggests that the magnitude of systemic antibody response has little, if any, correlation with the animal's apparent ability to counter the challenge of exposure to *M. paratuberculosis*.

An unexpected finding in the present study was the accumulation of mononuclear cells around intestinal nerves in 8 of 12 naturally infected sheep and in 5 of 14 experimental animals with histological evidence of paratuberculosis. These neural lesions resembled changes observed in early stages of leprosy and AFO's were detected within some of the mononuclear cells in 3 cases. There was no evidence of nerve involvement in the experimental sheep considered to be free of paratuberculosis lesions. This suggests that the neural lesions were associated with *M. paratuberculosis* infection.

Neural lesions are a fundamental feature of *M. leprae* infection in people. Nerve involvement in other mycobacterial infections has rarely been documented. Cases of granulomatous myelitis, radiculitis and neurits have been reported in infection with *M. avium* in parma wallabies (Schoon *et al.,* 1993). DNA of *M. paratuberculosis*, or some closely related *M. avium* spp, has been detected by PCR in cerebrospinal fluid from a human patient with neurosarcoidosis (El-Zaatari *et al.,* 1997b). The only neural lesions previously described in paratuberculous cattle, were characterised by infiltration of myenteric ganglions with globule leukocytes (Buergelt *et al.,* 1978a). Such lesions were not observed in the current study, probably due to either species variation in the host response to infection or variation of strain pathogenicity.

Of importance is the question of whether the intestinal nerves or the nerve-associated cells, as occurs in *M. leprae* infections in people, were the primary target of the inflammatory response. In the present study, the neural lesions were not detected in all animals with histological evidence of paratuberculosis, nor were they generalised.

Nerves run parallel to lymphatics and granulomatous inflammation of intestinal lymphatics is a feature of paratuberculosis. Thus, the neural lesions may represent an extension of local lymphangitis and the nerve involvement is probably secondary rather than primary. However, it is possible that nerves or the nerve-associated cells in the affected parts of intestine express stress-antigens (heat-shock proteins) that trigger an autoimune-like reaction. Furthermore, although AFO's were detected within the epi- and perineural mononuclear cells in only 3 sheep, the possibility of inflammatory response due to the presence of mycobaterial antigen(s) in the epineurium and perineurium in other cases cannot be excluded.

Regardless of the mechanisms underlying the development of neural lesions, the possibility of interference with nerve functions and subsequent contribution to the pathogenesis of paratuberculosis should be considered. An axonal degeneration in the sciatic nerves in mice inoculated into the hind foot pads with *M. avium* demonstrates that the ability to induce neural changes is not limited to infections with *M. leprae* (Kamala *et al.,* 1984). In the study cited, other mycobacterial species also showed growth in the foot pad but no lesions were seen in sciatic nerves. This suggests that mechanisms other than the multiplication of *M. avium* were likely to be involved in the generation of the nerve damage. In the current study, ultrastructural examination of the affected nerves was not conducted and the significance of the neural lesions remains to be determined.

In the present study, similar numbers of experimental sheep tested positive by the PCR on samples of ileum and ileocaecal lymph node were identified as infected by histology. This suggests that the PCR assay used in this study offers little advantage over histology in the routine post-mortem diagnosis of paratuberculosis. The 4 negative PCR results obtained in tissues from 4 animals with granulomatous lesions where AFO's could be seen microscopically are likely to represent false negative results, because other tissues from these sheep tested positive. Possible explanations include inadequate extraction of *M. paratuberculosis* from tissues, insufficient extraction of DNA from *M. paratuberculosis*, the presence of PCR inhibitors, or a combination of these. The sporadic detection of AFO's in faeces, usually from paratuberculous sheep in the advanced clinical stage, exemplifies the limited usefulness of this technique in the diagnosis of paratuberculosis.

In summary, the high incidence of clinical paratuberculosis in inoculated sheep and a close resemblance between histological lesions in experimentally and naturally infected animals suggest that the model presented here is suitable for the study of various aspects of *M. paratuberculosis* infection in sheep. The absence of histological lesions of paratuberculosis and negative PCR results in 7 experimentally infected sheep which had previously shown positive serological reactions, suggest that these animals had the ability to counter the challenge of exposure to the organism. The distinct aggregation of mononuclear cells around intestinal nerves in both naturally and experimentally infected paratuberculous sheep may represent an extension of local lymphangitis rather than neurotropic phenomenon. Further studies into the pathogenesis of neural lesions in paratuberculosis are required in order to determine their significance.

# Chapter 5

# Vaccination against paratuberculosis of lambs already infected experimentally with *Mycobacterium paratuberculosis*

## 5.1. Introduction

Programmes to control paratuberculosis are based on either vaccination, or the diagnosis and culling of infected animals. Vaccination has been reported to reduce mortality and faecal shedding of the organism in cattle, sheep, deer and goats (Sigurdsson, 1960; Doyle, 1964; Wilesmith, 1982; Saxegaard and Fodstad, 1985; Körmendy, 1994; Fawcett *et al.,* 1995), but most reports are incomplete and it is frequently difficult to determine if the beneficial effects are due to vaccination or to changes of management.

An increased host resistance to *Mycobacterium paratuberculosis* has previously been reported in sheep vaccinated prior to experimental infection (Nisbet *et. al.,* 1962; Juste *et al.,* 1994). However, in endemically infected herds many animals are expected to be exposed to the infection before vaccination (Chiodini *et al.,* 1984b). Since young animals are believed to be most susceptible to the infection (Chiodini *et al.,* 1984b), it has been recommended to vaccinate animals within the first month of their life.

The purpose of the study presented in this chapter was two-fold. Firstly, to assess the protective, or therapeutic, value of a live attenuated vaccine in sheep already exposed to *M. paratuberculosis.* Secondly, to provide blood samples and liver biopsy specimens from subclinically infected animals for further evaluation of the PCR assay, the development and initial evaluation of which were described in Chapters 2 and 3, respectively.

This chapter describes the effect of vaccination, using a commercial live attenuated vaccine, on the survival rate, severity of infection and systemic immune response in sheep already exposed to *M. paratuberculosis.* The application of the PCR for the detection of *M. paratuberculosis* in blood and liver biopsy specimens from sheep experi-

mentally infected with the organism is presented in Chapter 6.

## 5.2. Materials and methods

### 5.2.1. Animals and husbandry conditions

Forty-one crossbred lambs obtained from a flock where paratuberculosis had not previously been diagnosed, were randomly stratified for sex and weight into 3 groups. Two groups of 14 lambs, aged 1.0-1.5 months, were inoculated by stomach tube with 25 ml of inoculum containing approximately $4.4 \times 10^8$ *M. paratuberculosis* organisms. Two weeks later one group of 14 experimentally infected lambs was vaccinated by subcutaneous injection of 1 ml of live-attenuated vaccine (Neoparasec ®, Rhone Merieux, France, Batch 53E3101) in the right side of the neck. At the time of oral inoculation with *M. paratuberculosis*, a group of 13 untreated lambs (control) was transferred to a paddock adjacent to the grazing area that was designated for the infected-vaccinated (vaccinated) and infected-unvaccinated (unvaccinated) animals. Due to limitations on available space, it was not possible to include a group of vaccinated but uninoculated sheep.

The sheep in all 3 treatment groups were monitored twice per week throughout the duration of the trial for signs of ill health. In addition, they were weighed 2 weeks prior to oral inoculation with *M. paratuberculosis*, 2 weeks after the inoculation, and then successively at approximately 2-monthly intervals. Individuals showing signs of gradual loss of condition and progressive weakness of 3-4 weeks duration were removed from the group and killed by intravenous (i.v.) injection of pentobarbitone.

The parasitic burden in all 41 animals was controlled by oral administration of antihelmintic capsules (Extender, Captec, New Zealand), at approximately 3-monthly intervals. Nematode egg counts, performed at The Department of Veterinary Pathology and Public Health, Massey University, Palmerston North, New Zealand, on faeces collected initially on vaccination day, and then at approximately 2-monthly intervals, were used to monitor the effectiveness of parasite control. The experiment was terminated 53 weeks after inoculation, when all surviving experimental and control sheep were killed

by i.v. administration of pentobarbitone.

### 5.2.2. Inoculum

The ileum, and approximately one meter of the distal jejunum, collected during necropsy from 3 sheep with clinical paratuberculosis (confirmed by histology and PCR), were cut into 10-20 cm long segments. Aliquots of approximately 200 g of tissue were transferred to stomacher bags (Seward, UK) containing 200-400 ml phosphate buffered saline [PBS: 0.14 M NaCl, 8 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$; pH 7.2], homogenised in a stomacher for 20-30 min. In order to remove large particles, the mixture was filtered through gauze. Triplicate aliquots of 1 µl of a $10^{-5}$ dilution of the filtrate were stained by the Kinyuon method (Hendrickson and Krenz, 1991). Acid-fast organisms (AFO's) in each 1 µl aliquot were counted microscopically (40 x objective). The counts were averaged and 1 ml of inoculum was estimated to contain $9.7x10^6$ organisms ($1.6x10^6$ - $1.7x10^7$; 95%CI). The filtrate was stored at 4°C for 2 weeks. Immediately prior to use, the inoculum was thoroughly mixed.

### 5.2.3. Necropsy and histology

During post-mortem examination, gross changes consistent with paratuberculosis were observed and assessed subjectively as mild, moderate or severe. Samples for histological examination were collected as follows: ileum (1 sample of 5 cm, taken 1 cm from the ileocaecal valve), jejunum (1 sample of 5 cm, taken at the level of the caudal mesenteric lymph node), and 1 sample of the ileocaecal lymph node, caudal mesenteric lymph node, liver and kidney. Specimens of other tissues were collected only if required in the light of gross findings.

After fixation in 10% buffered formol saline, tissues were routinely processed, embedded in paraffin and sectioned at 4 µm. Of each tissue sample, 2 sections were stained with hematoxylin and eosin (H & E) and the remaining 2 by Ziehl-Neelsen's (ZN) method.

The sections were examined and aggregates composed of ten or more closely packed

macrophages were classified as microgranulomas. The microgranulomas were counted in 10 random fields of each section (10 x objective) and the total count from both sections of each tissue was divided by 2.

To determine the presence of AFO's, at least 50 high power fields (40 x objective) of each ZN-stained tissue section were examined. After this initial examination, the number of organisms per high power field was estimated. Since both the microgranulomas and AFO's were in some cases too numerous to count, a scoring system was adopted to facilitate statistical analysis of the magnitude of granulomatous inflammation and numbers of the organisms in tissues examined. The scoring system is presented in Table 5.1.

**Table 5.1. Scoring system of numbers of microgranulomas and acid-fast organism.**

| Number of AFO's per selected high power field (40xobjective) | Mean number of microgranulomas per 10 medium power fields (20xobjective) | Score |
|---|---|---|
| 0 | < 1 | 0 |
| 1 - 5 | 1 - 5 | 1 |
| 6 - 10 | 6 - 10 | 2 |
| 11 - 20 | 11 - 20 | 3 |
| 21 - 30 | 21 - 30 | 4 |
| 31 - 40 | 31 - 40 | 5 |
| 41 - 50 | 41 - 50 | 6 |
| 51 - 60 | 51 - 60 | 7 |
| 61 - 70 | 61 - 70 | 8 |
| 71 - 80 | 71 - 80 | 9 |
| 91 - 100 | 91 - 100 | 11 |
| >100 | >100 | 12 |

## 5.2.4. Sample collection and handling

***Blood***

Blood was collected from the jugular vein of each sheep into 4 ml plain evacuated tubes for serological tests and into 4 ml evacuated tubes containing lithium heparin for the interferon-γ (IFN-γ) assay. Blood samples were obtained initially on the day of oral inoculation with *M. paratuberculosis* and then 2, 5, 9, 18, 27, 36, 44 and 53 weeks after inoculation.

***Serum for serological testing***

After separation from the clot, samples of serum were initially frozen at -20°C for 2-7 days, then stored at -70°C for subsequent serological testing.

***Plasma for IFN-γ assay***

Preliminary results, presented in Appendix B.1, indicated that 10 μg Johnin purified protein derivate/1 ml of blood might be the optimal concentration for the IFN-γ assay. The processing of samples of blood was performed by an adaptation of the method described by Goff (1996). Each heparinised blood sample was processed within 6-7 h of collection by dispensing 1.5 ml aliquots into each of 2 polypropylene tubes. To one tube, 15 μl PBS containing 15 μg Johnin purified protein derivate (Johnin PPD) from *M. paratuberculosis* 316F (Central Veterinary Laboratory, Weybridge, England) was added, and 15 μl of PBS alone was added to the other (nil antigen control). The tubes were capped, gently mixed, and incubated overnight (17-19 h) in humidified atmosphere of 5% $CO_2$ at 37°C. After centrifugation at 1,800-2,400 g for 5-10 min, plasma was harvested and stored at -70°C for subsequent testing.

***Faecal specimens***

Faeces for nematode egg counts and microscopical examination for presence of AFO's were collected from the rectum of vaccinated, unvaccinated and control sheep on the day of oral inoculation with *M. paratuberculosis*, and then at approximately 2-monthly intervals.

***Tissues samples for PCR***

Immediately after euthanasia, and prior to necropsy, samples of ileum and ileocaecal lymph node for the PCR were collected from each sheep using clean, sterile instruments and containers. The processing of tissue specimens and extraction of DNA from these samples was performed as described in Chapter 2 (2.2.2.2) Each sample of extracted DNA was eluted in 120 μl of sterile, distilled water and 10 μl (approximate equivalent of 4 mg of tissue) was tested by the PCR.

### 5.2.5. Polymerase Chain Reaction

***Amplification***

Duplicate samples of DNA extracted from the ileum and ileocaecal lymph node were tested by the PCR. Each (10 µl) sample was amplified in 50 µl of PCR mixture using the Expand Long Template system (Boehringer Mannheim, Germany) under standard conditions described in Chapters 2 (2.2.3) and 4 (4.2.5). The reactions were performed using sterile procedures and following contamination-free guidelines, as described in Chapter 2 (2.2.3). For each set of reactions, positive (*M. paratuberculosis* DNA) and negative (water and reagents) controls were included.

***Analysis of PCR products***

The analysis of PCR products consisted of electrophoresis followed by visual examination of 10 µl aliquots of PCR products in 2% agarose gel as described in Chapter 2 (2.2.6). The identity of 400 base pair (bp) amplicons (products of the 90 and 91 primers) was confirmed by dot blot hybridisation with a 194-bp probe that was generated with primers JG1 and JG2 that bind internal to the primers 90 and 91 amplification product. The 194-bp probe was labelled with digoxigenin (DIG) (Boehringer Mannheim, Germany) and dot blot hybridisation was performed as described in Chapter 4 (4.2.5; see Appendix A.1 for details). All primers were manufactured by GIBCO BRL, New Zealand.

***Interpretation of PCR results***

The reactions were classified as positive, suspicious or negative using the interpretation scheme described in Chapter 4 (4.2.5). The rationale for such interpretation of the PCR results is described in Chapter 3 (3.2.4).

### 5.2.6. Immunological testing

***Serological testing***

Duplicate serum samples were assayed for antibody concentration using a commercial bovine enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's (CSL, Australia) recommendations (see Appendix A.2). The results were validated us-

ing criteria described in Chapter 4 (4.2.7). The optical density results (OD), read at 450 nm, were converted to antibody indices by dividing the mean OD of each serum by the mean OD of the plate positive control.

***Interferon-γassay***

Duplicate plasma samples were assayed for interferon-γ (IFN-γ) using a commercial bovine enzyme-linked immunosorbent assay (Bovigam™) according to the manufacturer's (CSL, Australia) recommendations (see Appendix B.2). The results of each plate were considered valid if the mean optical density (OD), read at 450 nm, of the negative controls was <0.13, the mean of the positive controls was > 0.7, and values of the positive controls had less than 30% deviation from their mean absorbance. Samples of plasma collected sequentially from an individual animal were tested on the same plate. Samples were re-tested when the coefficient of variation of their duplicates OD exceeded 30%. The results were initially expressed as OD measured at 450 nm. The OD results were converted to indices using the following formula:

$$\gamma\text{-IFN index} = \frac{\text{mean OD Johnin PPD stimulated samples - mean OD nil antigen samples}}{\text{mean OD plate positive controls - mean OD plate negative controls}}$$ (Bassey and Collins, 1997)

## 5.2.7. Microscopical examination of faecal smears for acid-fast organisms

Smears of faeces from infected-vaccinated, infected-unvaccinated and control sheep were stained by the Kinyuon method and examined microscopically (40 x objective). Results were considered positive when AFO's were present in clumps and their morphology closely resembled the appearance of organisms in a positive control sample from a sheep with histologically confirmed paratuberculosis. Samples with singular, non-clumped acid-fast bodies were classified as suspicious.

## 5.2.8. Haematology

Concentrations of haemoglobin, erythrocyte counts and white blood cells counts in heparinised blood samples were determined within 8 hours of collection, using the Cobos Minos *Vet* haematology analyser (Roche, France).

### 5.2.9. Statistical analysis

All statistical analyses were performed using the GraphPad Prism, version 2.01, program (GraphPad Software Incorporated, USA).

***Survival curves, PCR and histology data***

The survival curves of infected-vaccinated and infected-unvaccinated sheep were compared using the log-rank test. The Mann-Whitney test was employed to compare differences between the magnitude of the granulomatous inflammatory reaction in intestinal and mesenteric lymph node sections of these 2 groups of animals.

The degree of correlation between numbers of AFO's, magnitude of granulomatous reaction and PCR results was determined using the Spearman test. The PCR data were analysed using the Fisher exact test.

***Immunological data***

In order to normalise the distribution and equalise the variances of data, the antibody and IFN-γ indices were transformed, using Ln (antibody index) and Ln (IFN-γ index +1) transformations. The infected-vaccinated, infected-unvaccinated and control groups were compared using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Highly skewed data that could not be normalised, were analysed using the Kruskal-Wallis/Dunn's multiple comparison test.

***Haematological, faecal egg count and weight data***

Log-transformed (Ln) body weight and heamatological data of infected-vaccinated and infected-unvaccinated sheep were analysed by ANOVA/Tukey's multiple comparison test. The Kruskal-Wallis/Dunn's multiple comparison test was used to compare differences between the faecal egg counts in infected-vaccinated and infected-unvaccinated sheep.

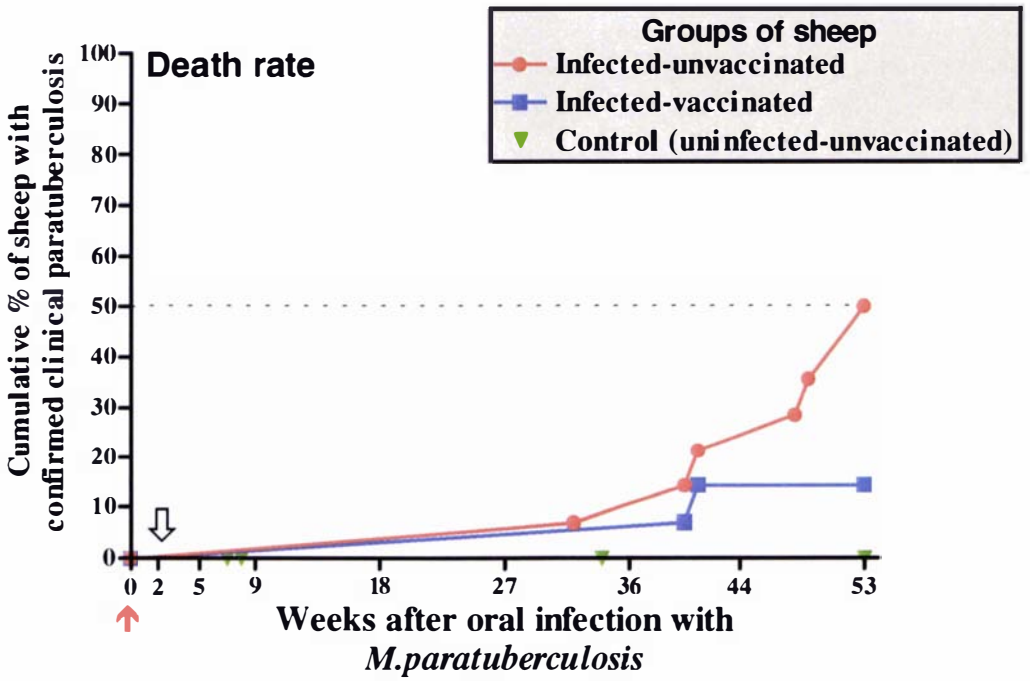
**Figure 5.1.** Death rate of 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis*, 14 infected but unvaccinated sheep and 13 uninfected-unvaccinated control sheep. Inoculation time is indicated by ↑ and vaccination by ⇩. The death rate of infected-vaccinated and infected-unvaccinated sheep was compared using the Logrank test (P = 0.0586).

## 5.3. Results

### 5.3.1. Death rate and gross pathology

The death rate of infected-vaccinated, infected-unvaccinated, and uninfected and unvaccinated (control) sheep is presented in Figure 5.1. Seven of 14 infected-unvaccinated animals and 2 of 14 infected-vaccinated sheep (P=0.0586) developed clinical signs suggestive of paratuberculosis and were sacrificed. Of 13 control sheep, 3 died of causes unrelated to paratuberculosis (see Appendix B.4).

**Figure 5.1.**




Necropsy findings in the 14 infected-vaccinated, 14 infected-unvaccinated and the remaining 11 control sheep are presented in Table 5.2. The infection status of 2 control sheep that died between the 5th and 9th weeks of the experiment was not determined because they were not available for post-mortem examinations. The subcutaneous vaccination site lesions, in all 14 infected-vaccinated animals, were white-grey, round, firm cystic structures that contained purulent, yellow-green, creamy material. The lesions, varying in size from 2 x 3 to 3 x 4 cm, extended deep into the subcutaneous fascia of the neck muscles. The right prescapular lymph nodes, draining the vaccination sites, were enlarged, firm, white-grey and nodular in all 14 vaccinated animals. On cut surface, scanty to numerous, small, pin-point white-grey foci were present in the cortex.

**Table 5.2. Gross pathology findings in 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis*, 14 infected but unvaccinated sheep and 11 uninfected and unvaccinated control sheep.**

| Gross lesions | | Number of sheep with lesions | | | | |
|---|---|---|---|---|---|---|
| | | infected-unvaccinated | | infected-vaccinated | | Control |
| | | Clinically affected n=7 | Clinically normal n=7 | Clinically affected n=2 | Clinically normal n=12 | n=11 |
| Body condition | good | - | 7 | - | 12 | 11 |
| | light | 1 | - | 1 | - | - |
| | poor | 6 | - | 1 | - | - |
| Thickening of terminal ileum | absent | 2 | 4 | - | 11 | 9 |
| | mild | 3 | 3 | 1 | 1 | 2 |
| | moderate | 1 | - | - | - | - |
| | severe | 1 | - | 1 | - | - |
| Enlargement of ICLN* | absent | 2 | 5 | - | 10 | 8 |
| | mild | 2 | 1 | - | - | 3 |
| | moderate | - | 1 | 1 | 2 | - |
| | severe | 3 | - | 1 | - | - |
| Serosal lymphatics | normal | 3 | 7 | 1 | 12 | 11 |
| | prominent | 4 | - | 1 | - | - |

ICLN*: ileocaecal lymph node

## 5.3.2. Histology

### 5.3.2.1. Experimentally infected vaccinated and unvaccinated sheep

***Ileum and jejunum***

Nine of 14 infected-unvaccinated sheep, 7 clinically affected and 2 clinically normal animals, had granulomatous lesions, composed of large, closely packed macrophages with abundant granular cytoplasm in the lamina propria of the distal ileum and jejunum. The magnitude of this lepromatous-type granulomatous reaction varied between sheep and was less severe in the jejunum. In 3 of these 9 animals, the lamina propria of the ileum was diffusely and heavily infiltrated with macrophages (Figure 5.3). The remaining 6 sheep had focal aggregates of macrophages that were predominantly located in the tips of ileal villi (Figure 5.4). Among the 9 sheep with granulomatous lesions in the ileal lamina propria, 5 had granulomas in the submucosa. The submucosal reaction ranged from intensive, diffuse infiltration composed of macrophages in one sheep to

**Figure 5.2.** Score of granulomas in the jejunal and ileal lamina propria, caudal mesenteric lymph node, ileocaecal lymph node and liver of 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis* and 14 infected but unvaccinated sheep. Bars indicate medians. The granuloma scores were analysed by Mann-Whitney test. Significant ($P<0.05$) differences between infected-unvaccinated and infected-vaccinated animals are indicated by *. CMLN - caudal mesenteric lymph node, ICLN - ileocaecal lymph node

moderate diffuse lymphoid cell infiltration accompanied by focal aggregates of macrophages in others.

In comparison, only 3 of 14 infected-vaccinated animals, 2 clinically affected and one clinically normal sheep, had scarce, small aggregates of large macrophages in the lamina propria of the distal ileum and/or jejunum (Figure 5.5). Submucosal focal aggregates of macrophages were evident in 2 of these 3 sheep.

Of the remaining 5 infected-unvaccinated and 11 infected-vaccinated sheep, one unvaccinated and 3 vaccinated animals had tuberculoid-like lesions, composed of focal areas of necrosis surrounded by macrophages, lymphocytes and fibroblasts, scattered between intestinal crypts of the jejunum or ileum (Figure 5.8).

As presented in Figure 5.2, the median number (score) of granulomas in the ileal and jeunal lamina propria was significantly greater in infected-unvaccinated than in infected-vaccinated sheep.

**Figure 5.2.**

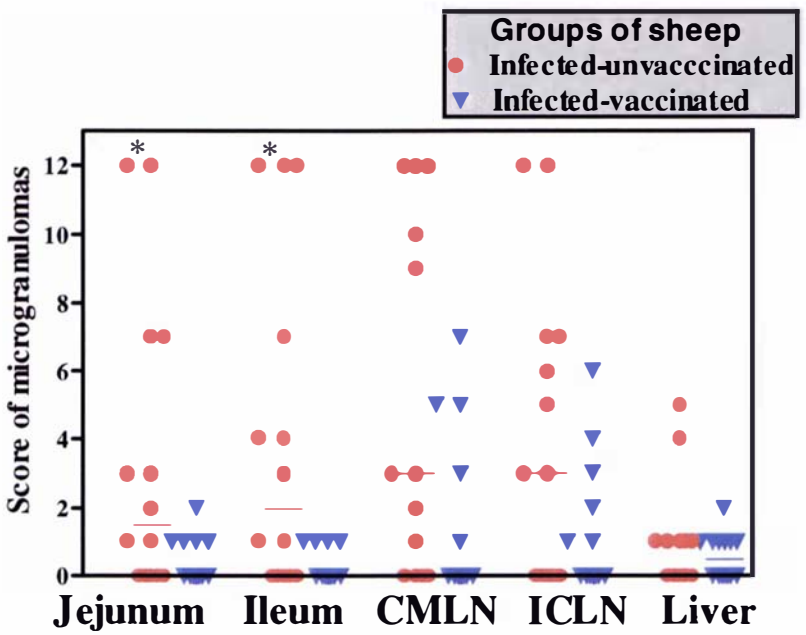


**Figure 5.3.** Small intestine (ileum) from a sheep infected orally with *M. paratuberculosis*. The lamina propria shows diffuse infiltration with macrophages. H & E x 130

Multinucleate giant cells were present in intestinal sections of one unvaccinated and 3 vaccinated sheep.

Nests of macrophages, containing AFO's, were detected in colonic sections of 2 clinically affected infected-unvaccinated sheep that had granulomatous inflammation in the ileum and jejunum.

**Figure 5.3.**

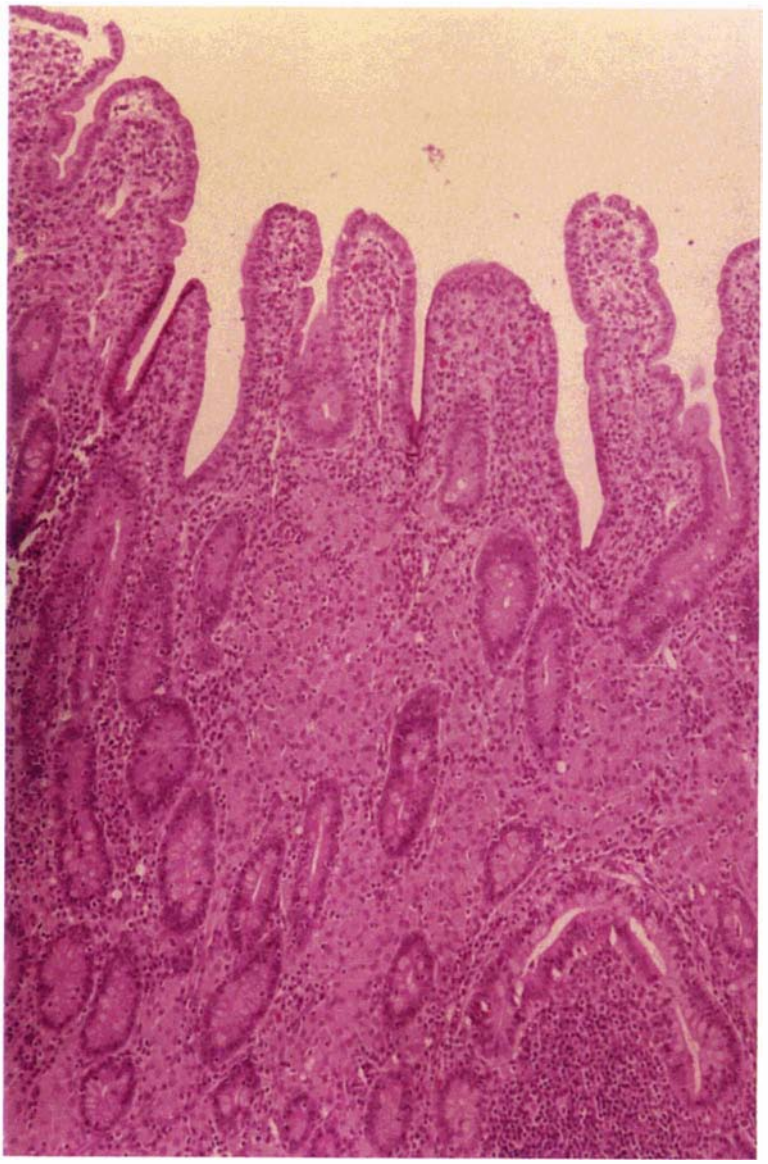


**Figure 5.4.** Small intestine (ileum) from a sheep infected orally with *M. paratuberculosis*. Aggregates of macrophages ⇑ predominantly located in the tips of ileal villi. H & E x 320

**Figure 5.5.** . Small intestine (ileum) from a sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis*. A small aggregate of macrophages ⇑ in the lamina propria. H & E x 430

**Figure 5.4.**

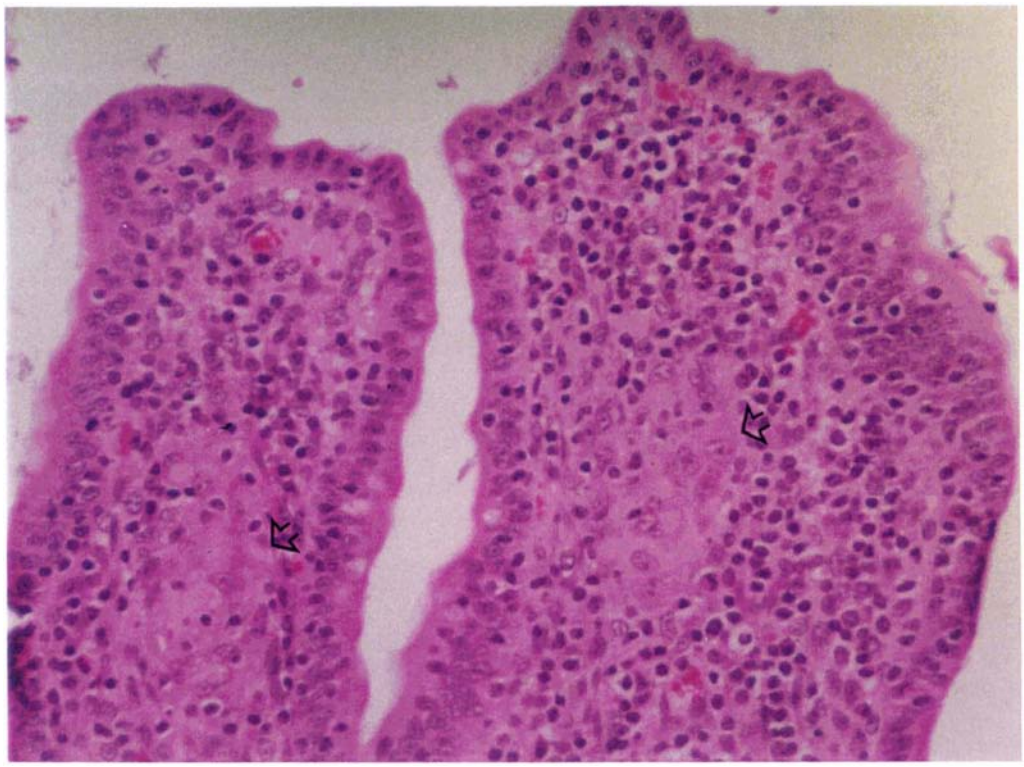
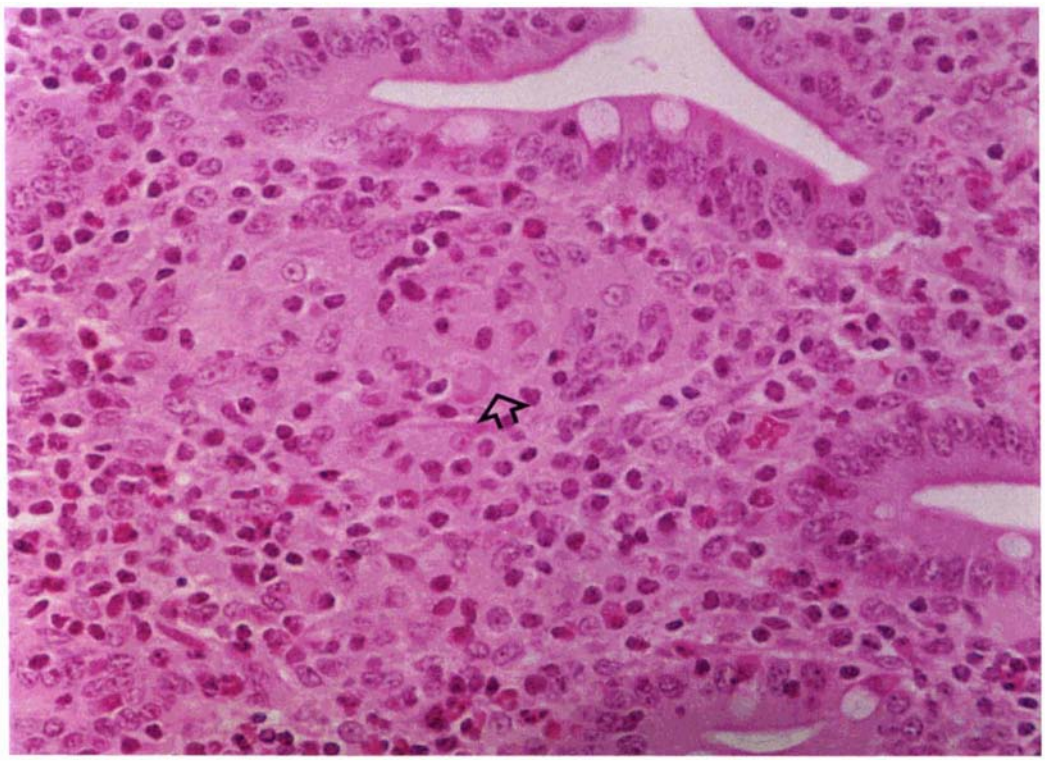


**Figure 5.5.**



AFO's were detected in some macrophages in the ileal mucosa of 7 infected-unvaccinated sheep, 6 clinically affected and one clinically normal animal. In 6 of these 7 sheep, 5 clinically affected and one clinically normal animal, the organisms were also evident, though in smaller numbers, in the jejunal mucosa. The AFO's were identified only in sheep with a lepromatous-type inflammatory reaction and the number (score) of organisms was closely correlated with the number (score) of microgranulomas in the ileal ($r=0.9657$, $P<0.0001$) and jejunal ($r=0.8570$, $P=0.0002$) lamina propria. No AFO's were detected in sections of intestine from any of the 14 infected-vaccinated sheep.

***Mesenteric lymph nodes***

In 10 infected-unvaccinated and 6 infected-vaccinated sheep, aggregates of macrophages were present in cortices of the ileocaecal lymph nodes and/or caudal mesenteric lymph nodes. One of these 10 unvaccinated sheep and one of the 6 vaccinated animals had no evidence of intestinal lesions. The number and size of lymph node microgranulomas varied between animals. In some cases as few as ten mononuclear cells constituted a microgranuloma, while in other cases the macrophages were too numerous to count. In one vaccinated and 2 unvaccinated sheep, the granulomas showed evidence of central necrosis. Multinucleate giant cells were evident in sections of mesenteric lymph node of 2 unvaccinated and 3 vaccinated sheep (Figure 5.6).

There was no significant difference between the median number (score) of microgranulomas in the ileocaecal lymph nodes and caudal mesenteric lymph nodes of unvaccinated and vaccinated sheep.

In 6 of 14 infected-unvaccinated sheep, AFO's were detected, though in small numbers, in the mesenteric lymph nodes. The AFO's were identified only in sheep with a lepromatous-type inflammatory reaction and the number (score) of the organisms was closely correlated with the number (score) of microgranulomas in both the caudal mesenteric lymph node ($r=0.8253$, $P=0.0005$) and iloecaecal lymph node ($r=0.7051$, $P=0.0049$). No AFO's were detected in the mesenteric lymph nodes of any of the 14 infected-vaccinated sheep.

***Liver***

Seven infected-unvaccinated sheep, comprising 4 clinically affected and 3 clinically normal animals, had aggregates of macrophages in the liver. Hepatic granulomas were also detected in 7 infected-vaccinated sheep, 2 clinically affected and 5 clinically normal animals. There was no significant difference between the median number of microgranulomas in the liver of unvaccinated and vaccinated sheep. Scanty AFO's were detected in hepatic microgranulomas of one unvaccinated sheep. No AFO's were detected in the liver of any of the 14 vaccinated sheep.

***Vaccination site lesions and draining lymph nodes***

In all 14 infected-vaccinated sheep the subcutaneous injection site lesion consisted of irregular coalescing areas of necrosis surrounded by an inner layer composed of macrophages, lymphocytes, multinucleate giant cells and sporadic neutrophils, and an outer zone of fibrous tissue. The necrotic centre, which in some areas was undergoing calcification, consisted of cellular debris with lipid vacuoles and occasional degenerate neutrophils. Scanty (1-10/HPF) AFO's were evident in the injection site lesions of all 14 vaccinates.

In addition, in 13 infected-vaccinated sheep, focal aggregates of macrophages and multinucleate giant cells were present, along with necrotic foci surrounded by macrophages, multinucleate giant cells, neutrophils and fibroblasts, in the cortices of prescapular lymph nodes draining the vaccination sites (Figures 5.7 and 5.9). The size and number of these lesions varied between animals. In 2 of the 13 vaccinated sheep, sparse AFO's were detected in sections of the prescapular lymph node. There was no evidence of granulomatous lesions in sections of prescapular lymph nodes from any of the infected-unvaccinated sheep.

### 5.3.2.2. Control sheep

None of the uninoculated and unvaccinated control sheep had granulomatous lesions or AFO's in sections of intestine, mesenteric lymph node, liver and prescapular lymph node.

**Figure 5.6.** Mesenteric lymph node from a sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis*. Multinucleate giant cells ⇑ in the cortex. H & E x 270

**Figure 5.7.** Prescapular lymph node, draining the vaccination site, from a sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis*. Multinucleate giant cells ⇑ in the cortex. H & E x 270

**Figure 5.6.**

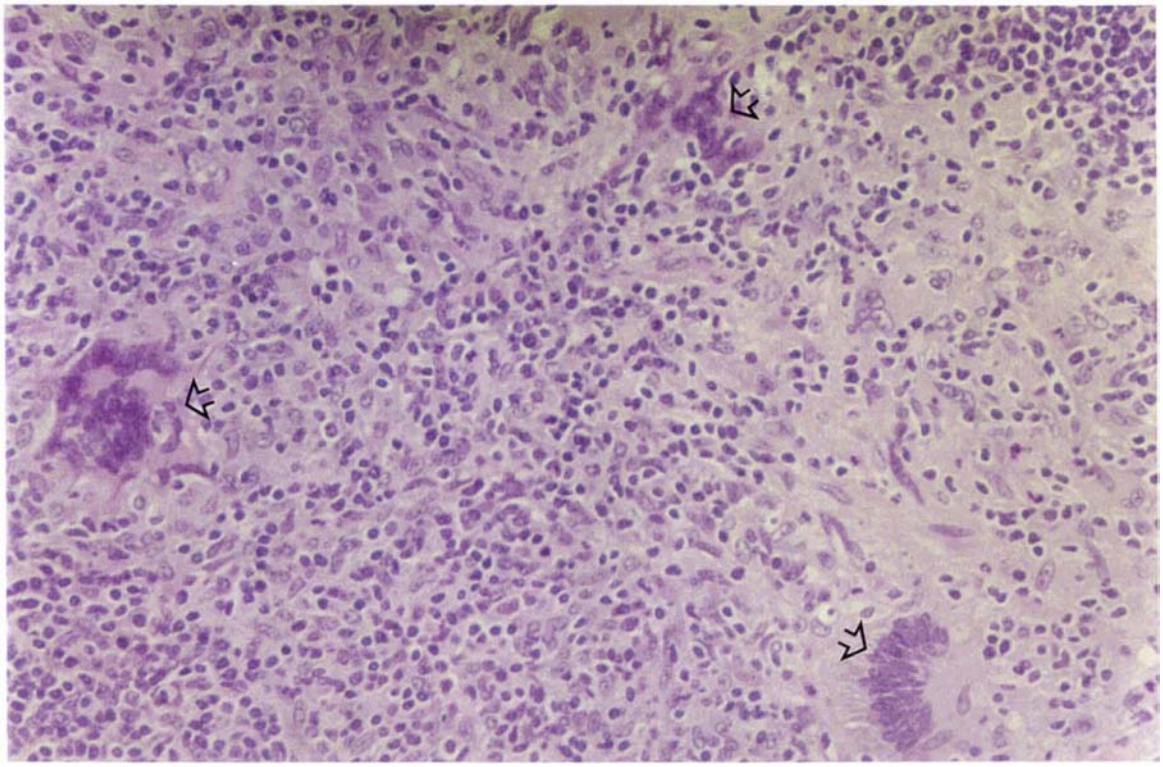


**Figure 5.7.**

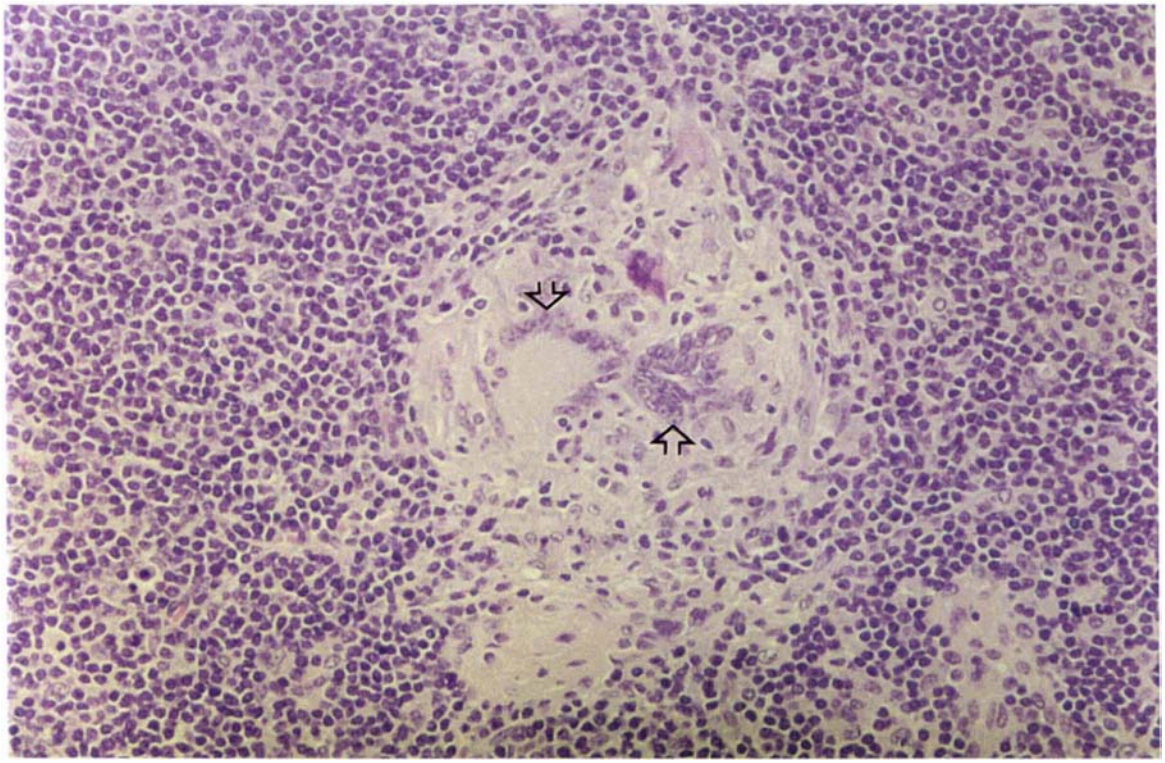


**Figure 5.8.** Small intestine from a sheep vaccinated against pratuberculosis 2 weeks after oral infection with *M. paratuberculosis*. Necrotic focus ⇑ surrounded by neutrophils, macrophages and fibroblasts in the lamina propria. (**C** - intestinal crypt) H & E x 320

**Figure 5.9.** Prescapular lymph node, draining the vaccination site, from a sheep vaccinated against pratuberculosis 2 weeks after oral infection with *M. paratuberculosis*. Necrotic focus ⇑ surrounded by neutrophils, macrophages and fibroblasts in the cortex. H & E x 270

**Figure 5.8.**

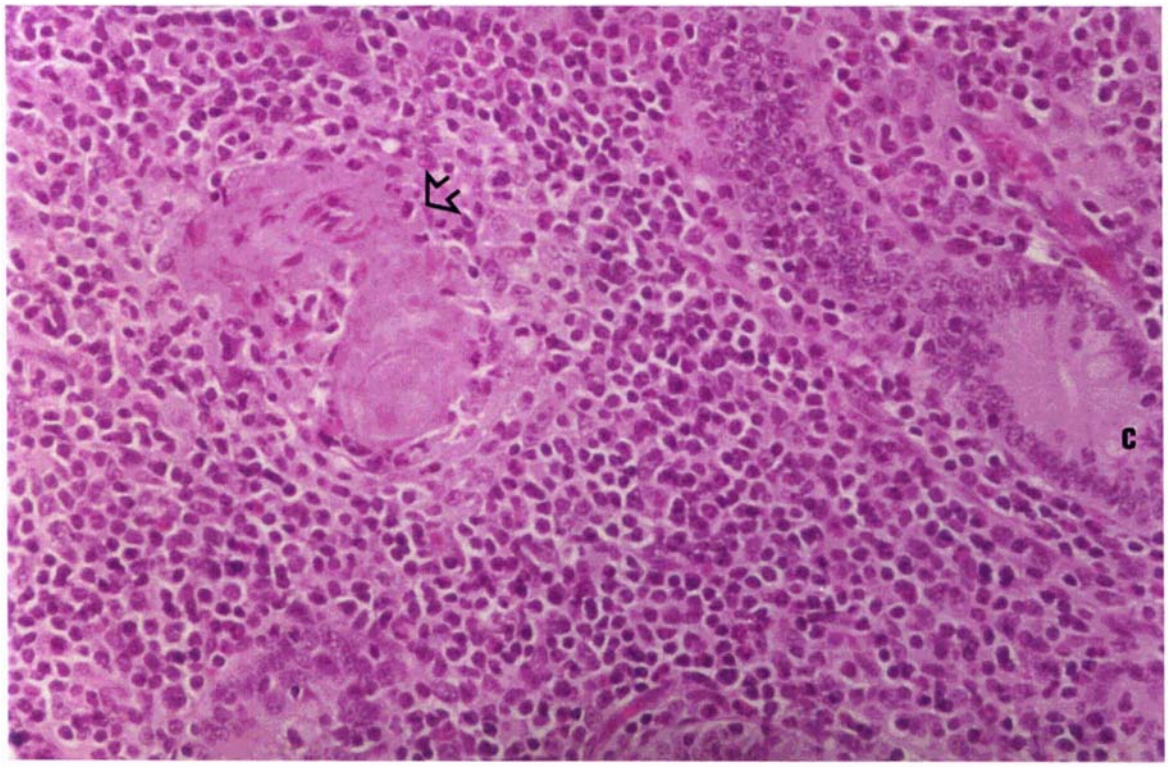
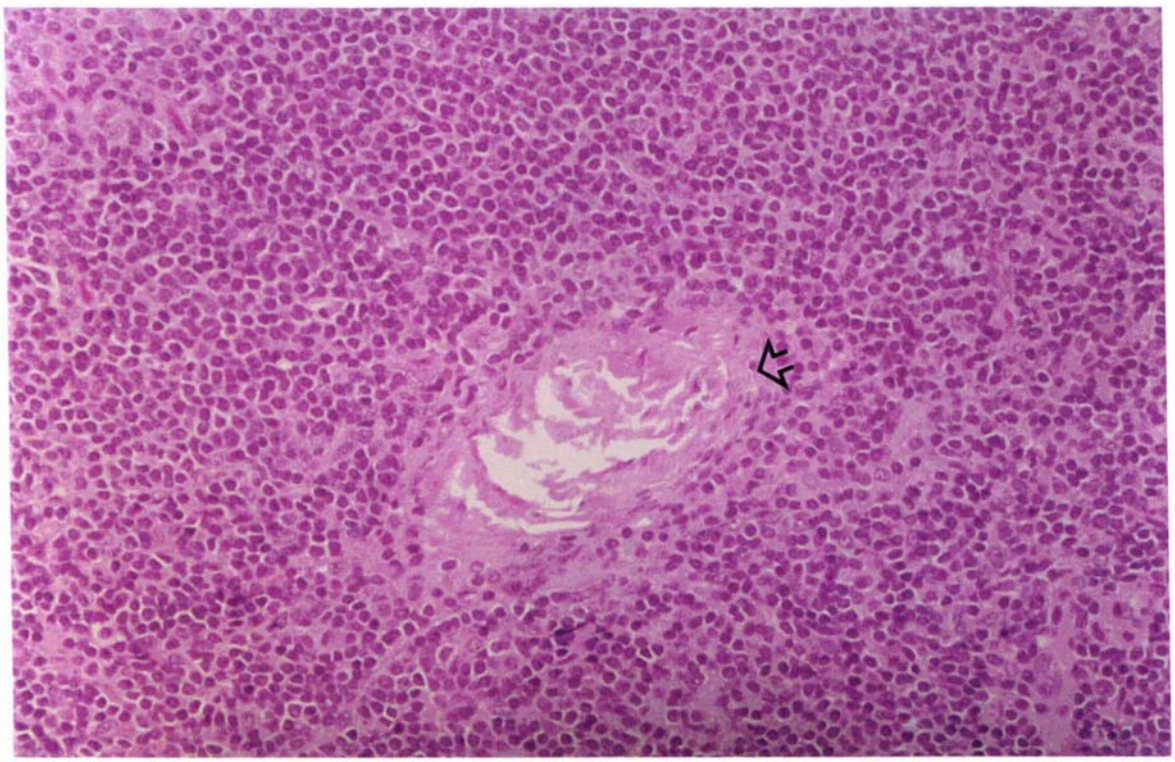



**Figure 5.9.**



### 5.3.3. Polymerase Chain Reaction

The PCR assay on duplicate samples of ileal DNA gave positive reactions in 2 of 14 infected-vaccinated sheep, both of which had granulomatous lesions without AFO's in sections of intestine, and in 8 of 14 infected-unvaccinated sheep (P=0.0461). Among the 8 infected-unvaccinated sheep, 7 had granulomatous lesions with AFO's in sections of the intestine and one had granulomatous lesions without AFO's. The PCR assay on duplicate samples of ileocaecal lymph node DNA was positive in 2 infected-vaccinated sheep, both of which had granulomatous lesions without AFO's in sections of ileocaecal lymph node, and in 7 infected-unvaccinated sheep (P=0.1032). Four of the 7 infected-unvaccinated sheep had granulomatous lesions with AFO's in the ileocaecal lymph nodes and 3 had equivocal lesions without AFO's. In total, 3 infected-vaccinated (21%) and 9 infected-unvaccinated (64%) sheep were tested positive by PCR. In addition, the PCR assay on duplicate samples of ileocaecal lymph node DNA gave 2 suspicious results. One of these suspicious reactions was obtained in one infected-unvaccinated sheep with granulomatous lesions without AFO's in the ileocaecal lymph node, and the other in one infected-unvaccinated sheep with no evidence of granulomatous lesions or AFO's in the ileocaecal lymph node. The results of the PCR and that derived from histology are presented in Appendix B.5.

In the infected-unvaccinated sheep the number (score) of AFO's was closely correlated with the PCR results in the ileum (r=0.6495, P=0.0119), but the correlation was weak in the ileocaecal lymph node (r=0.4493, P=0.1235).

None of the negative control sheep gave positive or suspicious reactions with the PCR on samples of ileum or ileocaecal lymph node.

### 5.3.4. Microscopical examination of faecal smears for acid-fast organisms

As presented in Table 5.3, clumps of AFO's were detected in faecal smears of 3 infected-unvaccinated sheep with clinical paratuberculosis that was later confirmed by histology and PCR. At all sampling points, none of the infected-vaccinated or control sheep had clumps of AFO's.

**Table 5.3. Results of microscopic examination of faeces for acid-fast organism in 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis*, 14 infected but unvaccinated sheep and 13 uninfected and unvaccinated control sheep compared with histology results.**

| Category of sheep | Sheep ID | Detection of AFO's in faeces: Weeks after infection 0 | 2 | 5 | 9 | 18 | 27 | 36 | 44 | 53 | | Histology results |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 32 | - | - | - | - | - | - | - | - | +/- | | - |
| Unvaccinated | 34 | - | - | - | - | - | - | - | + | + | # | + |
| | 36 | - | - | - | - | - | - | - | +/- | + | # | + |
| | 49 | - | - | - | - | - | - | nd | - | - | | - |
| | 51 | - | - | - | - | - | - | - | | | # | +/- |
| | 52 | - | - | - | - | - | - | - | - | - | | - |
| | 55 | - | - | - | - | - | - | | | | # | + |
| | 56 | - | - | - | - | - | - | - | - | - | | - |
| | 57 | - | - | - | - | - | - | - | +/- | - | | - |
| | 60 | - | - | - | - | - | - | - | + | | # | + |
| | 61 | - | - | - | - | - | - | - | | | # | + |
| | 67 | - | - | - | - | - | - | - | - | - | | +/- |
| | 68 | - | - | - | - | - | - | - | - | | # | + |
| | 69 | - | - | - | - | - | - | - | - | - | | + |
| Vaccinated | 33 | - | - | - | - | - | - | - | - | - | | - |
| | 40 | - | - | - | - | - | - | - | +/- | - | | - |
| | 43 | - | - | - | - | - | - | - | - | - | | +/- |
| | 44 | - | - | - | - | - | - | - | - | - | | (+/-) |
| | 46 | - | - | - | - | - | - | - | | | # | +/- |
| | 47 | - | - | - | - | - | - | nd | - | - | | - |
| | 53 | - | - | - | - | - | - | - | - | - | | - |
| | 54 | - | - | - | - | - | nd | - | - | - | | - |
| | 58 | - | - | - | - | - | - | - | - | - | | - |
| | 64 | - | - | - | - | - | - | - | | | # | +/- |
| | 65 | - | - | - | - | - | - | - | - | +/- | | - |
| | 66 | - | - | - | - | - | - | - | - | - | | - |
| | 70 | - | - | - | - | - | - | - | - | - | | - |
| | 71 | - | - | - | - | - | - | - | - | - | | - |
| Control | 31 | - | - | - | - | - | - | - | - | - | | - |
| | 35 | - | - | - | | | | | | | | nd |
| | 37 | - | - | - | - | - | - | - | - | - | | - |
| | 38 | - | - | - | - | - | - | - | - | - | | - |
| | 39 | - | - | - | - | - | - | - | - | - | | - |
| | 41 | | - | - | - | - | - | - | - | - | | - |
| | 42 | - | - | - | - | - | - | - | - | - | | - |
| | 45 | - | - | - | - | - | - | - | +/- | - | | - |
| | 48 | - | - | - | - | - | | | | | | - |
| | 50 | - | - | - | - | - | - | - | - | +/- | | - |
| | 59 | - | - | - | - | - | - | - | - | - | | - |
| | 62 | - | - | - | | | | | | | | nd |
| | 63 | - | - | - | - | - | - | - | - | - | | - |

**Microscopic examination of faeces:**

+ clumps of acid-fast organisms in faeces

+/- individual acid-fast bodies in faeces

- no acid-fast bodies detected in faeces

**Histology results** (presence or absence of lesions consistent with paratubeculosis in the ileum):

+ granulomatous lesions of lepromatous-like morphology with AFO's

+/- granulomatous lesions of lepromatous-like morphology without AFO's (equivocal lesions)

(+/-) lesions showing central necrosis in which AFO's were not detected (equivocal lesions of tuberculoid-like morphology)

- neither lepromatous- nor tuberculoid-like lesions detected (no AFO's)

\# clinical case

**nd** not done

### 5.3.5. Immunological testing

***IFN-γ and antibody response in vaccinated and unvaccinated sheep infected experimentally with M. paratuberculosis***

The mean IFN-γ indices (in response to Johnin PPD) in blood samples, and mean antibody indices in serum samples, of 14 infected-unvaccinated, 14 infected-vaccinated and 13 control sheep are presented in Figure 5.10. There was no change in IFN-γ and antibody levels in the control group throughout the duration of the experiment. In infected-vaccinated sheep, an initial rise in both the IFN-γ response and antibody titre was observed in the 5th week post-infection. In infected-unvaccinated sheep, no increase in IFN-γ response was detected until 18 weeks after inoculation, and no increase in antibody level was detected until 27 weeks. Between the 5th and 53rd week post-inoculation antibody concentrations measured in the infected-vaccinated group were significantly ($P<0.05$) greater than those in the infected-unvaccinated sheep. During this period, the mean IFN-γ indices were also higher in the infected-vaccinated sheep than those in the infected-unvaccinated sheep. There was significant difference between the mean production of IFN-γ in these two groups in the 9th and 27th week post-infection.

***Relationship between IFN-γ concentration and antibody titre and the presence of AFO's in tissues of unvaccinated sheep infected experimentally with M. paratuberculosis***

The IFN-γ and serum antibody responses in the 7 infected-unvaccinated sheep in which AFO's were not detected (AFO-ve), and in the 7 infected-unvaccinated sheep in which AFO's were detected (AFO+ve), are presented in Figure 5.11. The IFN-γ response in the first group of sheep was high, and between the 18th and 44th week post-inoculation varied significantly from that measured in the control sheep. In contrast, the magnitude of IFN-γ production in the group of AFO+ve infected-unvaccinated sheep was low, and differed significantly from that measured in the control sheep only at 44 weeks post-infection. There was a significant difference ($P<0.05$) between the mean IFN-γ indices in AFO-ve and AFO+ve infected-unvaccinated sheep 18 weeks after inoculation.

**Figure 5.10.** IFN-γ production in response to Johnin PPD in blood samples and antibody indices in serum samples of 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosi*s, 14 infected but unvaccinated sheep and 13 uninfected-unvaccinated control sheep. Inoculation time is indicated by ⇑ and vaccination by ⇩. During the experiment, the numbers of sheep in both treatment groups were declining. Due to progressive loss of condition, sheep were killed at the following stages: one infected-unvaccinated sheep between the 27th and 36th week; 2 infected-vaccinated and 2 infected-unvaccinated animals between the 36th and 44th week and 2 infected-unvaccinated sheep between the 44th and 53rd weeks postinoculation. Of the 13 control sheep, 3 died of causes unrelated to paratuberculosis, 2 between the 5th and 9th week, and one 34 weeks after initiation of the experiment. Data is presented as mean ±SEM. The results were log-transformed and analysed by ANOVA/Tukey's multiple comparison test. Significant differences ($P<0.05$) between the control and treatment groups are indicated by *, whereas (*) indicates significant differences between treatment groups. Very significant ($P<0.01$) and extremely significant results ($P<0.001$) are represented by ** and ***, respectively. (See Appendices B.3.1 and B.3.2 for details)

The pattern of antibody production was similar in AFO-ve and AFO+ve infected-unvaccinated sheep and there was no significant difference between the median antibody indices of the 2 groups.

**Figure 5.10.**

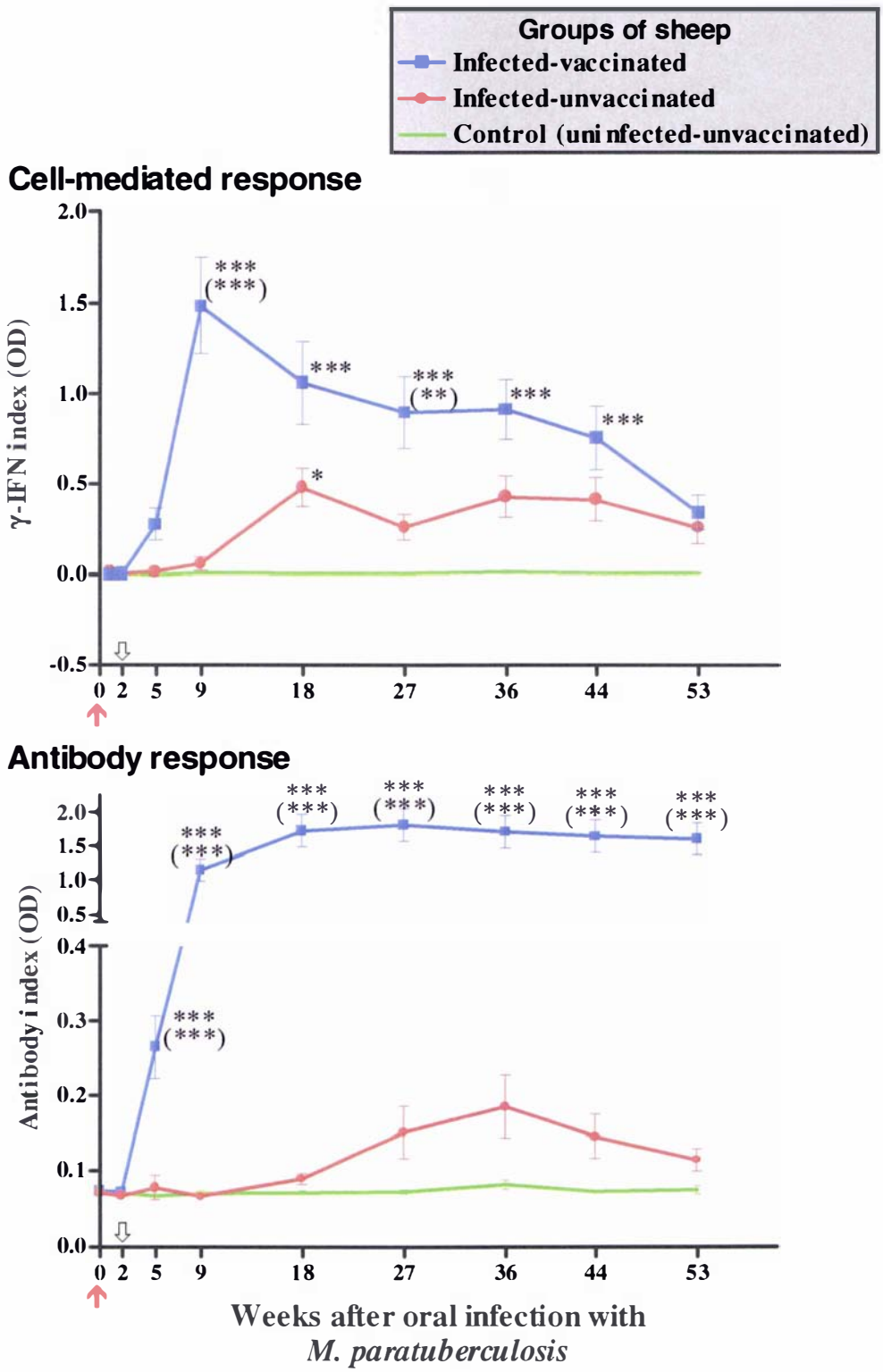
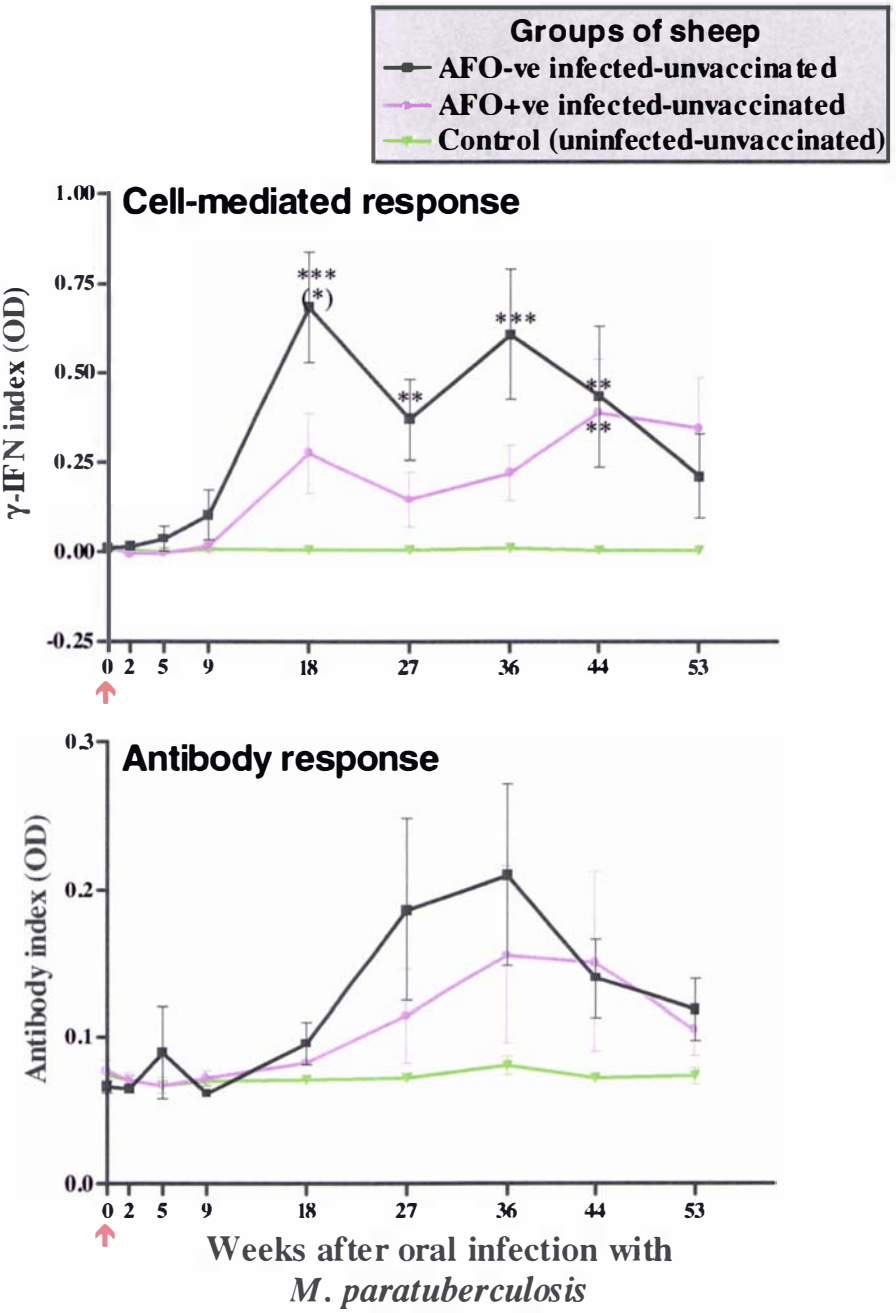


**Figure 5.11.** IFN-γ production in response to Johnin PPD in blood samples and antibody indices in serum samples of 14 sheep infected orally with *M. paratuberculosis* and 13 uninfected-unvaccinated controls. In 7 experimentally infected sheep acid-fast organisms were detected microscopically in sections of tissues examined (AFO's +ve sheep), while in the remaining 7 sheep AFO's were not detected (AFO's -ve sheep). Inoculation time is indicated by ↟. During the experiment the numbers of the experimentally infected but unvaccinated sheep were declining. Due to progressive loss of condition, sheep were killed at the following stages: one AFO+ve sheep between the 27th and 36th week; one AFO+ve sheep and one AFO-ve sheep between the 36th and 44th week and 2 AFO+ve sheep between the 44th and 53rd week postinoculation. Of the 13 control sheep, 3 died of causes unrelated to paratuberculosis, 2 between the 5th and 9th week, and one 34 weeks after initiation of the experiment. Results are presented as mean ± SEM. The IFN-γ data were log-transformed and analysed by ANOVA/Tukey's multiple comparison test. Antibody data were compared using the Kruskal-Wallis/Dunn's multiple comparison test. Significant differences ($P<0.05$) between the control and treatment groups are indicated by *, whereas (*) indicates significant differences between treatment groups. Respectively, very significant ($P<0.01$) and extremely significant results ($P<0.001$) are represented by ** and ***, respectively. (See Appendices B.3.3 and B.3.4 for details)

**Figure 5.11.**




### 5.3.6. Weight gain and faecal egg count

The weight gain, growth rate and faecal egg count data obtained from 14 infected-vaccinated, 14 infected-unvaccinated and 13 control (non-infected and unvaccinated) sheep are presented in Figure 5.12 and 5.13, respectively. There was no significant difference between the weight gain, growth rate and faecal egg count in infected-

**Figure 5.12.** Weight gain and growth rate in 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis,* 14 infected but unvaccinated sheep and 13 uninfected-unvaccinated control sheep. During the experiment, the numbers of animals in all groups were declining (see Figure 5.1). Inoculation time is indicated by ⇑ and vaccination by ⇩. Data is presented as mean ±SEM. The weight gain data of infected-vaccinated and infected-unvaccinated sheep were log-transformed and analysed by ANOVA, while the growth rate data of these 2 groups were compared using the Kruskal-Wallis/Dunn's multiple comparison test. At all sampling times there was no significant difference between these 2 groups ($P > 0.05$). Because the control sheep were kept separate from the infected groups, the data obtained from this control group, though presented here, were not compared statistically with the data from infected-vaccinated and infected-unvaccinated groups. (see Appendices B.3.5 and B.3.6 for details)

vaccinated and infected-unvaccinated sheep. Because the control sheep were kept separate from the infected groups the data obtained from this control group, though presented here, were not compared statistically with the data from infected-vaccinated and infected-unvaccinated groups.

**Figure 5.12.**

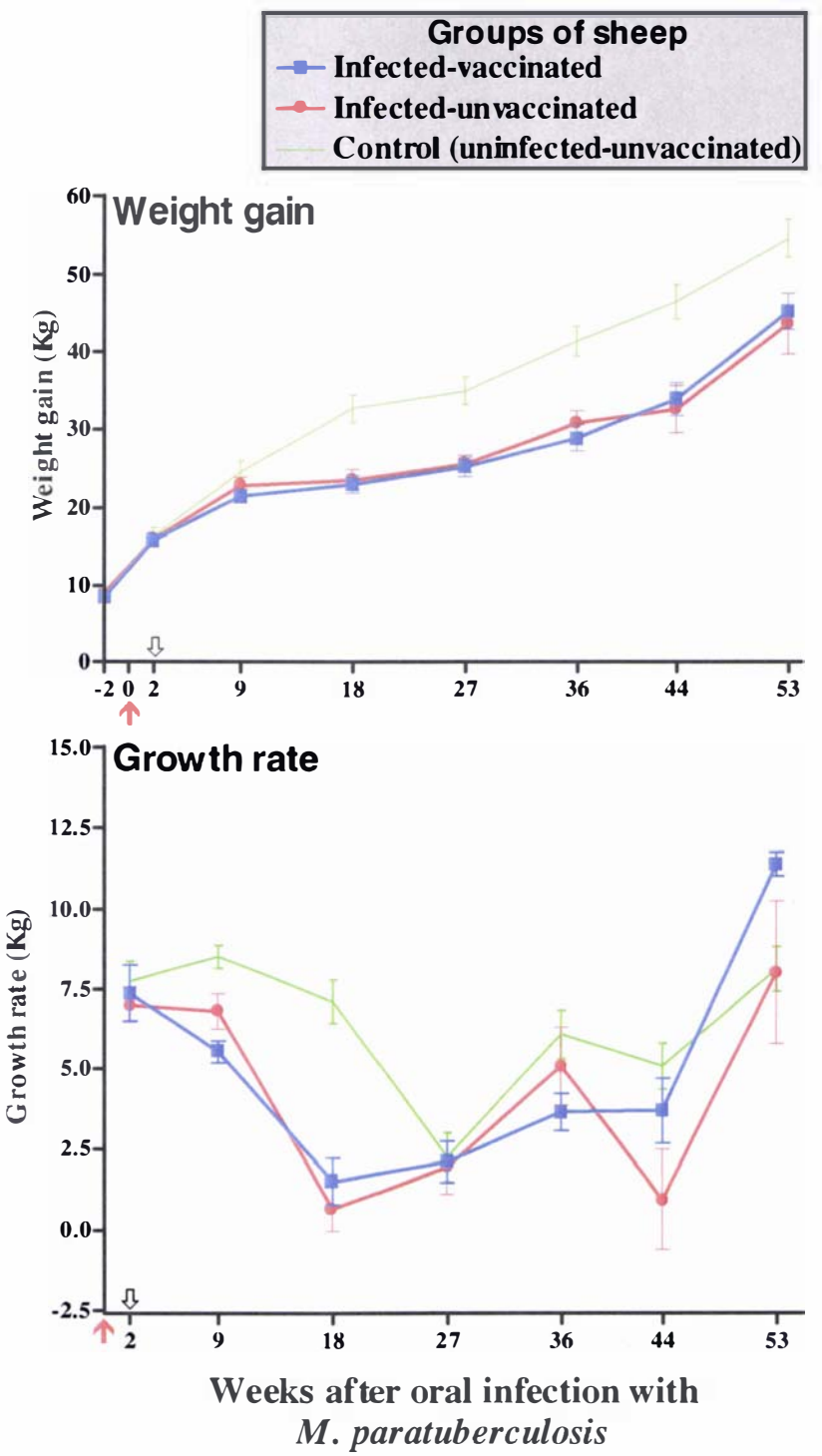
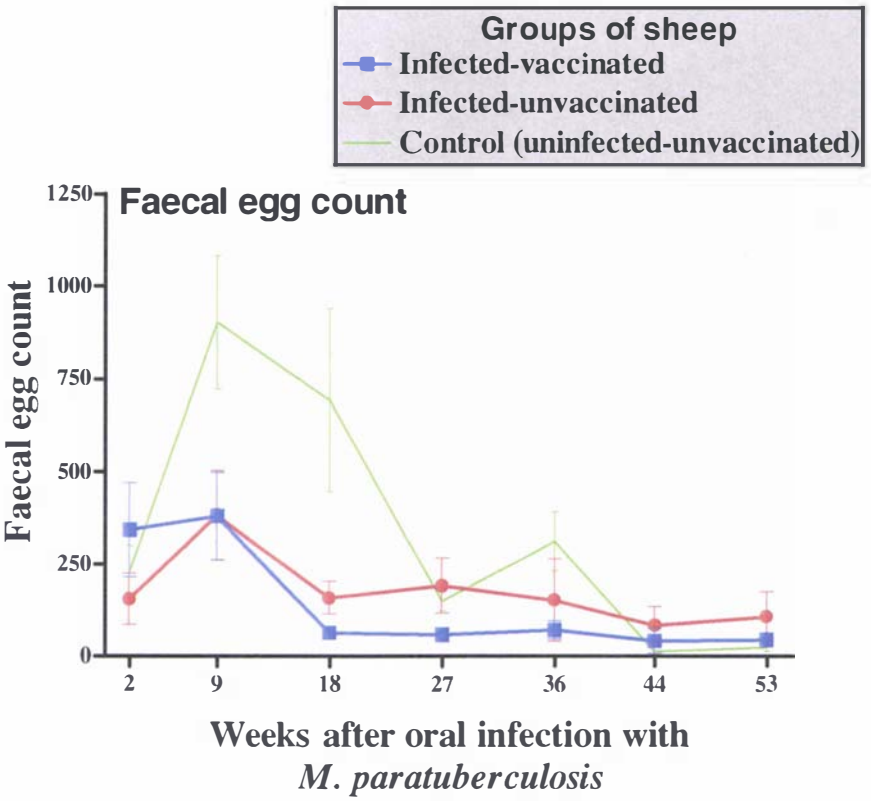


**Figure 5.13.** Nematode egg count in faecal samples of 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis,* 14 infected but unvaccinated sheep and 13 uninfected-unvaccinated control sheep. During the experiment, the numbers of animals in all groups were declining (see Figure 5.1.). Data is presented as means ±SEM. The results of infected-vaccinated and infected-unvaccinated sheep were analysed by the Kruskal-Wallis/Dunn's multiple comparison test. At all sampling times there was no significant difference between these 2 groups ($P > 0.05$). Because the control sheep were kept separate from the infected groups, the data obtained from this control group, though presented here, were not compared statistically with the data from infected-vaccinated and infected-unvaccinated groups. (See Appendix B.3.7 for details)

**Figure 5.13.**




### 5.3.7. Haematology

***Haematological values in vaccinated and unvaccinated sheep infected experimentally with M. paratuberculosis***

As presented in Figure 5.14, nine weeks post-infection the mean concentration of haemoglobin and the mean erythrocyte count were significantly lower ($P<0.05$) in infected-vaccinated sheep than in infected-unvaccinated animals. At all sampling points there was no significant difference between the mean total leukocyte count in infected-vaccinated sheep and that measured in infected-unvaccinated animals.

***Relationship between haematological values and the presence of AFO's in tissues of unvaccinated sheep infected experimentally with M. paratuberculosis***

The mean concentration of haemoglobin, the mean erythrocyte count, and the mean total leukocyte count in the 7 infected-unvaccinated sheep in tissues of which AFO's were detected, and in the 7 infected-unvaccinated sheep in tissues of which organisms were not detected, are presented in Figure 5.15. At all sampling points there was no

**Figure 5.14.** Erythrocyte count, haemoglobin concentration and total leukocyte count in blood samples of 14 sheep vaccinated against paratuberculosis 2 weeks after oral infection with *M. paratuberculosis,* 14 infected but unvaccinated sheep and 13 uninfected-unvaccinated control sheep. During the experiment, the numbers of animals in all groups were declining (see Figure 5.1). Inoculation time is indicated by ⇑ and vaccination by ⇩. Data is presented as mean ±SEM. The haematological data of infected-vaccinated and infected-unvaccinated sheep were log-transformed and analysed by ANOVA/Tukey's multiple comparison test. Significant differences ($P<0.05$) between these 2 groups are indicated by *. Because the control sheep were kept separate from the infected groups, the data obtained from this control group, though presented here, were not compared statistically with the data from infected-vaccinated and infected-unvaccinated groups. (See Appendices B.3.8, B.3.9 and B.3.10 for details)

significant difference between haematological values in these 2 groups of infected-unvaccinated sheep.

**Figure 5.14.**

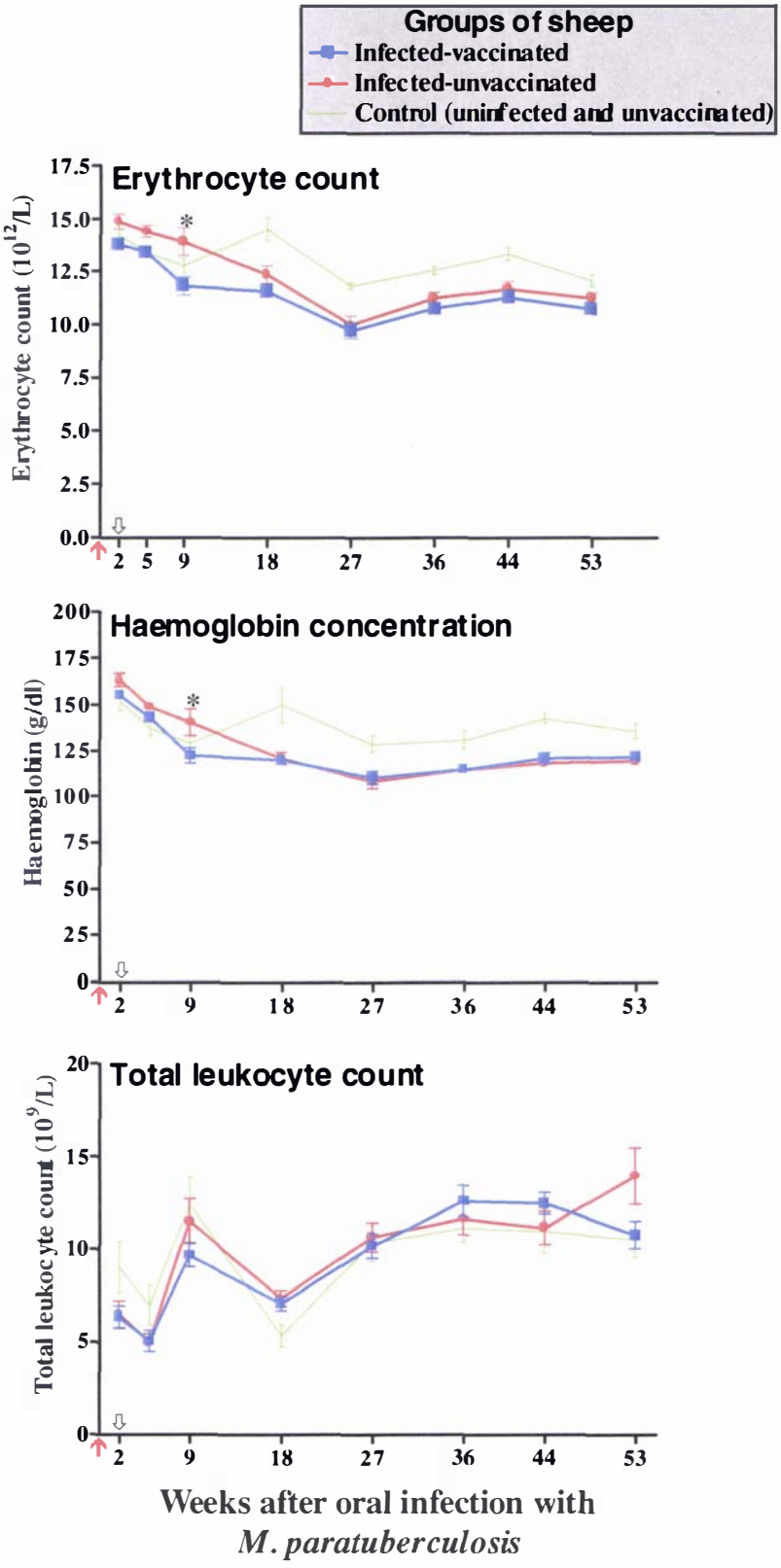
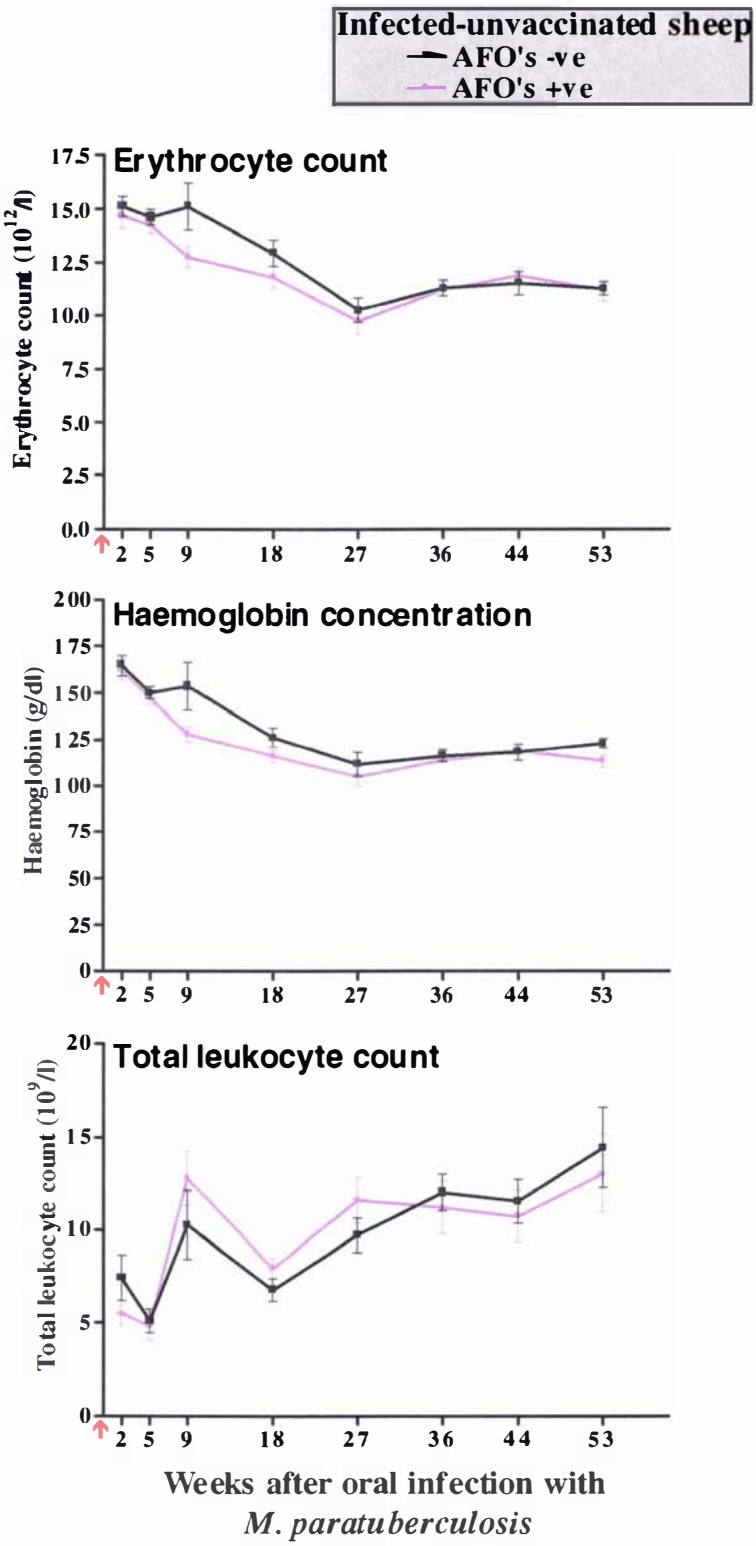


**Figure 5.15.** Erythrocyte count, haemoglobin concentration and total leukocyte count in blood samples of 14 sheep infected orally with *M. paratuberculosis*. In 7 experimentally infected sheep acid-fast organisms were detected microscopically in sections of tissues examined (AFO's +ve sheep), while in the remaining 7 sheep AFO's were not detected (AFO's -ve sheep). Inoculation time is indicated by ↑. During the experiment, the numbers of sheep in both groups were declining. Due to progressive loss of condition, sheep were killed at the following stages: one AFO's +ve sheep between the 27th and 36th week; one AFO's +ve sheep and one AFO's -ve sheep between the 36th and 44th week and 2 AFO's +ve sheep between the 44th and 53rd week postinoculation. Results are presented as mean ± SEM. The data were log-transformed and analysed by ANOVA/Tukey's multiple comparison test. At all sampling times there was no significant difference between these 2 groups ($P > 0.05$). (See Appendices B.3.11, B.3.12 and B.3.13 for details)

**Figure 5.15.**


Infected-unvaccinated sheep
AFO's -ve
AFO's +ve
Erythrocyte count
Erythrocyte count ($10^{12}$/l)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
2 5 9 18 27 36 44 53
Haemoglobin concentration
Haemoglobin (g/dl)
200
175
150
125
100
75
50
25
0
2 5 9 18 27 36 44 53
Total leukocyte count
Total leukocyte count ($10^9$/l)
20
15
10
5
0
2 5 9 18 27 36 44 53
Weeks after oral infection with
*M. paratuberculosis*

## 5.4. Discussion

In the longitudinal study presented here, 7 of 14 infected-unvaccinated (unvaccinated) sheep and only 2 of 14 infected-vaccinated (vaccinated) sheep developed clinical signs suggestive of paratuberculosis. AFO's were present in intestinal and/or mesenteric lymph node sections of 7 unvaccinated animals but none were detected in the intestine or mesenteric lymph node of the 14 vaccinated sheep. The PCR assay on ileum samples gave positive reactions in 2 vaccinated (14%) and 8 unvaccinated sheep (57%). The target DNA was also identified in ileocaecal lymph node samples of 2 vaccinates (14%) and 7 unvaccinated sheep (50%). In total, 3 vaccinated (21%) and 9 unvaccinated (64%) sheep tested positive by PCR. Clumps of AFO's were only detected in faecal smears of 3 unvaccinated, clinically affected sheep. Furthermore, the magnitude of granulomatous inflammation in the jejunal and ileal lamina propria was less in vaccinated than in unvaccinated sheep. These data indicate that vaccination of lambs with live-attenuated vaccine 2 weeks after oral inoculation stimulated the host response against *M. paratuberculosis* and reduced the severity of infection.

The results of study presented here are in contrast with a previous study where vaccination of sheep already infected with *M. paratuberculosis* did not modify the infection (Gilmour *et al.,* 1965). There are several possible explanations for this discrepancy. Firstly, vaccination and oral inoculation regimes in the experiment reported by Gilmour *et al.* (1965) were different to the study presented here. In the work by Gilmour *et al.* (1965), 8-week-old lambs were dosed orally once per week for 10 weeks, with a total dose of approximately $10^8$ viable units of *M. paratuberculosis.* One month after the last dose half of the infected sheep were vaccinated subcutaneously with heat-killed vaccine. In the current study, lambs, aged 1.0-1.5 months, were orally inoculated only once with approximately $4.4 \times 10^8$ *M. paratuberculosis* organisms and 2 weeks later half of the infected lambs were vaccinated subcutaneously with live-attenuated vaccine. Secondly, both ovine and bovine isolates were used to infect sheep in the study by Gilmour *et al.* (1965), while in the present study the inoculum contained *M. paratuberculosis* of ovine origin and the organisms were never cultured on artificial media. Thirdly, due to low incidence of intestinal infections in the unvaccinated group, the transmission of infection in the experiment

reported by Gilmour *et al.* (1965) was not particularly successful and the results were therefore rather inconclusive.

The mode of action of paratuberculosis vaccines is poorly understood. It has been postulated that early development and regression of granulomas is associated with the elimination of infection in vaccinated sheep (Juste *et al.*, 1994). In the current study the presence of granulomatous mesenteric lymphadenitis detected at necropsy in the vaccinated sheep suggests that the intestinal lesions may have been more severe early in the infection, as lesions in mesenteric lymph nodes are a sequel to intestinal infection. Since the magnitude of granulomatous inflammation in the jejunal and ileal lamina propria was less in the vaccinated group than in the unvaccinated group, this could be interpreted as an indication of early regression of infection in the intestine of vaccinated sheep. Alternatively, the granulomatous mesenteric lymphadenitis in the vaccinated sheep could have been caused by the vaccine-strain of *M. paratuberculosis* or by some other agent. However, it is impossible to be definitive because in-contact control groups of uninfected vaccinated and unvaccinated sheep were not able to be included in the trial.

The microgranulomas with AFO's observed in unvaccinated animals were composed of large, closely packed macrophages with abundant granular cytoplasm, and had lepromatous-type morphology. These lesions resembled most cases described by Carrigan and Seaman (1990), Clarke and Little (1996) and type 3a and 3b lesions reported by Perez *et al.* (1996). In contrast, the inflammatory response in vaccinated sheep and in unvaccinated animals with no apparent AFO's was more typically a delayed type hypersensitivity (DTH) reaction with necrosis and multinucleate giant cells. These tuberculoid-like microgranulomas were similar to lesions observed at vaccination sites and regional prescapular lymph nodes. It has previously been noted that in the intestine of vaccinated lambs there may be predominantly tuberculoid-type lesions with numerous multinucleate giant cells (Nisbet *et al.*, 1962). A direct relationship between DTH and clearance of *M. paratuberculosis* has been reported in a resistant (C57/B10) strain of mice (Chiodini and Buergelt, 1993). Due to the relatively low number of animals involved in the present study it is impossible to draw conclusions on the significance of caseous necrotic lesions in intestinal and mesenteric lymph nodes of sheep which appeared to control the infection.

Nevertheless, the absence of the organism, as determined by histology and PCR, in sheep with tuberculoid-like lesions, and the presence of lepromatous lesions in sheep which were unable to control the infection, suggest that either different mechanisms or other agents are involved in the development of each histological form.

The lack of significant difference between the growth rate of vaccinated and unvaccinated animals indicates that vaccination did not have a negative effect, but nor did it have a positive effect on this aspect of animal production in sheep already infected with *M. paratuberculosis.* Due to limited grazing space, a group of non-infected but vaccinated sheep was not included in this study and the effect of vaccination on the weight parameters in such a group of animals was not determined.

Although vaccination of lambs with live-attenuated vaccine 2 weeks after oral inoculation reduced the severity of infection and did not have a deleterious effect on the growth rate of vaccinated animals the vaccination site lesions ranged from 2 x 3 to 3 x 4 cm one year after vaccination. In spite of careful injection technique the lesions extended beyond the subcutaneous fascia of the neck muscles. The prescapular lymph nodes, draining the vaccination sites, were enlarged, firm, white-grey and on cut surface scanty to numerous, small, pin-point white-grey foci were present in the cortex. In addition, severe localised reaction after accidental self-inoculation present a human health risk (Björnsson *et al.,* 1971). In order to minimise these side effects, alternative formulations or routes of vaccination should be investigated.

In sheep infected orally with *M. paratuberculosis* and 2 weeks later vaccinated against paratuberculosis, a simultaneous and early increase in serum antibody levels and IFN-γ production was observed 5 weeks after oral infection (3 weeks postvaccination). In contrast, a rise in IFN-γ production within the group of 14 infected but unvaccinated animals was not detected until 18 weeks after oral inoculation with *M. paratuberculosis*, and an antibody response was not evident until 27 weeks after infection. In addition, the IFN-γ and antibody production was greater in the vaccinated group than in the unvaccinated sheep. These data indicate that vaccination is effective in early stimulation of a strong humoral and cell-mediated immune response.

An early antibody response in sheep vaccinated parenterally and delayed response in animals infected orally with *M. paratuberculosis*, has been previously reported (Juste *et al.,* 1994). The authors postulated that the lag time between the oral infection and detection of an immune response could be due to the development of partial tolerance, or unresponsiveness, to mycobacterial antigens. There is evidence that *M. paratuberculosis* suppresses the delayed hypersensitivity reaction in mice (Kishima *et al.,* 1991). Alzuherri *et al.* (1997) have reported a decreased expression of molecules involved in antigen presentation on the surface of intestinal ovine macrophages infected with *M. paratuberculosis*. Furthermore, glycolipid compounds of several mycobacterial species (reviewed by Reiner, 1994), including *M. avium* serovar 2 (formerly known as *M. paratuberculosis* strain 18) inhibit microbicidal functions of macrophages (Hines *et al.,* 1996). There is also a decreased expression of MHC class II and co-stimulatory molecules in mycobacteria-laden macrophages (Wadee *et al.,* 1995; Mohagheghpour *et al.,* 1997). These observations are compatible with the hypothesis of partial tolerance as a mechanism by which mycobacteria avoid the immune reaction. In the current study, parenteral vaccination resulted in an early and efficient presentation of mycobacterial antigens. It is likely that the early onset and high magnitude of the immune response in infected-vaccinated sheep was responsible for the ability of a significant proportion of these animals to control the infection.

In unvaccinated sheep inoculated with *M. paratuberculosis*, the cell-mediated response was detected earlier than the humoral reaction. This agrees with a previously reported observation that detection of the cellular reaction precedes detectable antibody production in experimentally infected cattle (Lepper *et al.,* 1989). In contrast, parenteral vaccination, 2 weeks after oral challenge with *M. paratuberculosis*, triggered a simultaneous antibody and cell-mediated response. It is known that the differentiation into Th1 and Th2 cells and subsequent development of cell-mediated or antibody responses may be affected by the dose, type and form of antigen (Bretscher, 1992; Mosmann and Sad, 1996; Elhay and Andersen, 1997), type of adjuvant (Elhay and Andersen, 1997) and the route of immunisation (Xu-Amano *et al.,* 1992; Xu-Amano *et al.,* 1994). Therefore it is possible that the parallel development of both antibody and cell-mediated response in the vaccinated group might have been due to the high concentration of antigen being administered both via the oral and parenteral

route.

The essential role of IFN-γ in defence against mycobacteria in mice is well documented (Cooper *et al.,* 1993; Kamijo *et al.,* 1993; Flesch *et al.,* 1995). It has recently been reported that expression of the IFN-γ gene was significantly higher in samples of ileum and caecal lymph node from subclinically infected cows than from cows with clinical paratuberculosis (Sweeney *et al.,* 1998). In the present study, the magnitude of IFN-γ response in blood in the group of experimentally infected but unvaccinated sheep in which AFO's were detected, was diminished in comparison with experimentally infected animals in which AFO's were not detected. This supports the hypothesis of Wentnik *et al.* (1993) that the capacity to control the infection is directly related to the magnitude of systemic cell-mediated response measured by skin test. In contrast to IFN-γ production, both the magnitude and pattern of the antibody response were similar in the group of infected-unvaccinated sheep in which AFO's were detected and in the group of infected-unvaccinated sheep in which AFO's were not detected. This is in agreement with the suggestion that antibodies play little, if any, role in immunity against mycobacteria (Collins, 1988).

A number of investigators have reported inhibition of human erythroid colony formation by IFN-γ *in vitro* (Zoumbos *et al.,* 1984; Mamus *et al.,* 1985; Raefsky *et al.,* 1985; Means and Krantz, 1991; Means *et al.,* 1992). It is also known that neopterin, a compound produced by human monocytes/macrophages upon stimulation with IFN-γ, inversely correlates with haemoglobin levels in various diseases (Denz *et. al.,* 1990; Biemba *et al.,* 1998). In the present study, the mean concentration of haemoglobin and the mean erythrocyte count were significantly lower ($P<0.05$) in infected-vaccinated sheep than in infected-unvaccinated animals 9 weeks post-infection. This coincided with a peak of IFN-γ response in the first group of animals. The possibility that the strong IFN-γ response might have contributed to a decrease in haemoglobin levels and to reduction in the number of erythrocytes in infected-vaccinated sheep 9 weeks post-infection cannot be ruled out. However, it is impossible to draw conclusions since these haematological values are undergoing physiological alterations in young animals. During the early stage of the present study, a declining trend for haemoglobin levels and numbers of erythrocytes was evident in all groups of lambs.

## Summary

Vaccination of lambs with live-attenuated vaccine 2 weeks after inoculation with *M. paratuberculosis* led to a reduced mycobacterial burden as indicated by histology and the PCR on ileum samples, and triggered early and strong humoral and cell-mediated immune responses. This immune response is likely to be associated with the reduced levels of intestinal infection and reduced faecal shedding of *M. paratuberculosis* in vaccinated sheep.

The diminished IFN-γ response in infected but unvaccinated sheep in which AFO's were detected, suggests that there is a positive relationship between the magnitude of systemic cell-mediated response and animal's ability to control the infection.

Sheep that appeared to control the infection tended to develop tuberculoid-type granulomatous lesions with few, if any, AFO's, while lepromatous-type lesions with abundant AFO's were detected in sheep that developed clinical signs. This suggests that either different mechanisms or other agents are involved in the development of each histological form.

The experimental model presented here proved to be a useful tool for the assessment of the protective value of the live-attenuated vaccine in sheep already infected with *M. paratuberculosis*.

# Chapter 6

# Application of the polymerase chain reaction assay for the detection of *Mycobacterium paratuberculosis* to blood and liver biopsy specimens from sheep experimentally infected with the organism

## 6.1. Introduction

Definitive diagnosis of paratuberculosis is based on identification of *Mycobacterium paratuberculosis* in faeces, intestines or mesenteric lymph nodes (Merkal, 1984). However, this requires 9-12 weeks and the sensitivity of the technique is only about 10% in sheep (Carrigan and Seaman, 1990). Consequently, attempts have been focused on the development of alternative methods to identify the organism in clinical specimens. The detection of *M. paratuberculosis* DNA by the polymerase chain reaction (PCR) in samples of blood and liver from a significant proportion of sheep with advanced clinical paratuberculosis, as described in Chapter 4, has raised some hope for the use of a PCR-based test in the diagnosis of paratuberculosis.

In order to further evaluate the PCR assay in subclinically infected animals a longitudinal study was designed. This chapter describes the application of the PCR assay to liver biopsy specimens and sequentially collected blood samples from sheep orally infected with *M. paratuberculosis*. The blood and liver biopsy PCR results are compared with those derived from histology and PCR on samples of ileum and ileocaecal lymph node collected at the time of necropsy.

## 6.2. Materials and methods

### 6.2.1. Animals and handling

The source of experimental sheep, the husbandry conditions under which they were

maintained, and the dose of *M. paratuberculosis* used to infect the sheep orally have been described in detail in Chapter 5 and will be only briefly summarised here.

Twenty-eight of 41 lambs, aged 1.0-1.5 month, were orally inoculated with approximately $4.4x10^8$ *M. paratuberculosis* organisms. Two weeks later 14 of these 28 lambs were vaccinated by subcutaneous injection of 1 ml of live-attenuated vaccine (Rhone Merieux, France). At the time of oral inoculation a control group of 13 untreated lambs was transferred onto a paddock adjacent to the grazing area that was designated for the infected-vaccinated (vaccinated) and infected-unvaccinated (unvaccinated) animals. Between the 32nd and 53rd week after inoculation, 7 infected-unvaccinated and 2 infected-vaccinated animals developed clinical paratuberculosis. All of these clinical cases were sacrificed by intravenous injection of pentobarbitone and submitted for necropsy. Three of 13 controls died in the early stage of the experiment for causes unrelated to paratuberculosis (see Appendix B.4). The experiment was terminated 53 weeks after inoculation with *M. paratuberculosis*, when all experimental and control sheep that had survived to the end of the experiment were killed by intravenous administration of pentobarbitone.

### 6.2.2. Infection status of animals

The infection status of each sheep, except 2 control animals that were not available for post-mortem examination (see Appendix B.4), was determined at the time of necropsy by histology and duplicate PCR tests on DNA extracted from samples of ileum and ileocaecal lymph node, as described in Chapter 5 (5.3.2 and 5.3.3).

### 6.2.3. Sample collection and processing

***Blood***

Blood was collected from the jugular vein of each sheep into 4 ml tubes containing ethylenediaminetetra-acetic acid disodium (EDTA). The blood samples for PCR were obtained initially on the day of oral inoculation with *M. paratuberculosis* and then 2, 5, 9, 18, 27, 36, 44 and 53 weeks postinoculation. Isolation of leukocytes from all blood samples was performed within 6-8 h of collection by a method described in Chapter 3 (3.2.3).

***Liver biopsy***

Liver biopsy specimens were collected 32 weeks after oral inoculation. Prior to the surgical procedure the area of the right abdomen and thorax of each sheep was clipped and disinfected with aqueous hibitane. A true-cut liver biopsy specimen was collected, under intravenous barbiturate-induced general anaesthesia, from each animal using a probe that was introduced into the abdominal cavity through a small (1-3 cm) incision in the skin in the middle of the right 11th intercostal space. After collection, each biopsy specimen was transferred to a 15 ml Falcon tube containing 2 ml of buffer [100 mM NaCl, 25 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, 0.5% sodium dodecyl sulphate] and stored on ice prior to further processing. Within 6-8 hr of collection, all liver specimens were weighed, the volume of buffer adjusted to 1 ml/100 mg of tissue, and then 200 µg (20 µl) of Proteinase K was added. The mixture was incubated overnight at 50°C.

## 6.2.4. DNA extraction

The DNA was extracted from isolated peripheral blood leukocytes and specimens of liver using the Proteinase K/CTAB-phenol-chloroform method described in Chapter 2 (2.2.2.2). Each sample of hepatic DNA was eluted in 540 µl of water and 45 µl (approximate equivalents of 4 mg of tissue) was tested by the PCR. Each sample of leukocyte DNA was re-suspended in 100 µl of water and 45 µl (equivalent of approximately 0.5 ml of blood) was tested by the PCR.

## 6.2.5. Polymerase Chain Reaction

***Amplification***

Duplicate samples of DNA extracted from the ileum and ileocaecal lymph node were tested as described in Chapter 5 (5.2.5). Each sample of hepatic and leukocyte DNA was amplified using the Expand Long Template system (Boehringer Mannheim, Germany) under standard conditions described in Chapter 2 (2.2.3) in a reaction mixture containing 2 µM of each primer (90 and 91), 50 mM of Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 0.5 mM each dNTP, and 5.25 units of Taq/Pwo polymerase mixture (Boehringer Mannheim, Germany) in a total volume of 100 µl,

covered by mineral oil. For each set of reactions, positive (*M. paratuberculosis* DNA) and negative (water and reagents) controls were included. The reactions were performed following contamination-free guidelines as described in Chapter 2 (2.2.3).

***Analysis of PCR product***

The analysis of PCR products consisted of electrophoresis followed by visual examination of 10 µl aliquots of PCR products in 2% agarose gel as described in Chapter 2 (2.2.6). The identity of 400 base pair (bp) amplicons (products of the 90 and 91 primers) was confirmed by dot blot hybridisation with a digoxigenin-labelled 194-bp probe (product of primers JG1 and JG2) using the method described in Chapter 4 (4.2.5, see Appendix B.1 for details).

***Interpretation of PCR results***

The reactions obtained in duplicate samples of solid tissue DNA were classified as positive, suspicious or negative using the interpretation scheme described in Chapter 4 (4.2.5). The reaction obtained in a single sample of blood DNA was classified as positive, if a band of the right size (400-bp) was identified on a gel, the hybridisation results were concordant, and no signal was obtained with the negative control. The reaction was considered as suspicious if a band of the right size was not visible on a gel but a hybridisation signal was obtained in the dot blot analysis. The rationale for such interpretation of PCR results is described in Chapter 3 (3.2.4).

### 6.2.6. Statistical analysis

The number of experimentally infected sheep in which the presence of infection was determined by histology was compared with the number of sheep tested positive by the PCR on samples of ileum and ileocaecal lymph node using the Fisher exact test (GraphPad Prism, version 2.01, GraphPad Software Incorporated, USA).

## 6.3. Results

The results of the PCR assay on duplicate samples of hepatic DNA, single samples of peripheral blood leukocyte DNA and duplicate samples of ileal and ileocaecal lymph

node DNA are summarised in Table 6.1. The DNA samples were obtained from 14 sheep infected with *M. paratuberculosis*, 14 sheep vaccinated against paratuberculosis 2 weeks after infection and 13 control sheep.

### 6.3.1. PCR on samples of ileum and ileocaecal lymph node

There was no significant difference between the number of vaccinated and unvaccinated sheep identified as infected by histology and the number of animals that tested positive by the PCR on samples of ileum and ileocaecal lymph node ($P>0.05$).

The PCR assay on duplicate samples of ileal DNA gave positive reactions in 2 of 14 infected-vaccinated sheep, both of which had equivocal lesions without acid-fast organisms (AFO's) in sections of the intestine, and in 8 of 14 infected-unvaccinated sheep. Among the 8 PCR+ve infected-unvaccinated sheep, 7 had granulomatous lesions with AFO's in sections of the intestine and one had equivocal lesions.

The PCR assay on duplicate samples of ileocaecal lymph node DNA was positive in 2 of 14 infected-vaccinated sheep, both of which had equivocal lesions in the ileocaecal lymph nodes, and in 7 of 14 infected-unvaccinated sheep. Four of the 7 PCR+ve infected-unvaccinated sheep had granulomatous lesions with AFO's in the ileocaecal lymph nodes and 3 had equivocal lesions. Two sheep tested positive by the ileocaecal lymph node-based PCR, one infected-vaccinated and one infected-unvaccinated, were negative in the ileum-based PCR. These 2 sheep had equivocal lesions both in the intestine and ileocaecal lymph nodes. In addition, the PCR assay on samples of ileocaecal lymph node DNA gave 2 suspicious results. One of these suspicious reactions was obtained in one infected-unvaccinated sheep with granulomatous lesions without AFO's in the ileocaecal lymph node, and the other in one infected-unvaccinated sheep with no evidence of granulomatous lesions or AFO's in the ileocaecal lymph node. The lymph node-based PCR assay was negative in one infected-unvaccinated sheep that had scanty AFO's in the mesenteric lymph node.

None of the negative control sheep gave a positive or suspicious reaction in the PCR on samples of ileum and ileocaecal lymph node.

The results of the PCR on selected samples of tissues are presented in Figure 6.1.

**Table 6.1. Results of the PCR assay on duplicate samples of hepatic (liver biopsy) DNA, single samples of peripheral blood leukocyte DNA and duplicate samples of ileal and ileocaecal lymph node DNA compared with results obtained by histology. The DNA samples were obtained from 14 sheep infected experimentally with *M. paratuberculosis*, 14 sheep vaccinated against paratuberculosis 2 weeks after infection and 13 uninfected and unvaccinated controls**

| | Sheep ID | *Liver biopsy PCR results | Blood PCR results: Weeks after inoculation 0 | 2 | 5 | 9 | 18 | 27 | 36 | 44 | 53 | | Histology and PCR results at the time of necropsy: Histology ileum | Histology ICLN | PCR ileum | PCR ICLN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 32 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| U | 34 | - | - | - | - | - | - | - | - | - | - | # | + | + | + | - |
| n | 36 | - | - | - | - | - | - | - | - | - | - | # | + | + | + | + |
| v | 49 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| a | 51 | -/+ | - | - | - | - | - | - | - | | | # | +/- | +/- | - | + |
| c | 52 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| c | 55 | + | - | - | - | - | - | - | | | | # | + | + | + | + |
| i | 56 | ND | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| n | 57 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| a | 60 | - | - | - | - | - | - | - | - | - | | # | + | + | + | + |
| t | 61 | - | - | - | - | - | + | - | - | | | # | + | +/- | + | + |
| e | 67 | - | - | - | - | - | -/+ | - | - | - | - | | +/- | (+/-) | + | + |
| d | 68 | - | - | - | - | - | - | - | - | - | | # | + | +/- | + | +/- |
| | 69 | + | - | - | - | - | - | + | - | - | - | | + | + | + | + |
| | 33 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| | 40 | - | - | - | - | - | - | -/+ | - | - | - | | - | (+/-) | - | - |
| V | 43 | - | - | - | - | - | + | - | - | - | - | | +/- | +/- | - | + |
| a | 44 | - | - | - | - | - | + | - | - | - | - | | (+/-) | - | - | - |
| c | 46 | - | - | - | - | - | - | - | - | | | # | +/- | +/- | + | + |
| c | 47 | - | - | - | - | - | -/+ | - | - | - | - | | - | - | - | - |
| i | 53 | - | - | - | - | - | - | - | - | - | - | | - | +/- | - | - |
| n | 54 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| a | 58 | - | - | - | - | - | - | - | - | - | - | | - | - | - | +/- |
| t | 64 | - | - | - | - | - | - | - | - | | | # | +/- | +/- | + | - |
| e | 65 | - | - | - | - | - | - | - | - | - | - | | - | (+/-) | - | - |
| d | 66 | - | - | - | - | - | + | - | - | - | - | | - | - | - | - |
| | 70 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| | 71 | - | - | - | - | - | -/+ | - | - | - | - | | - | - | - | - |
| | 35 | ND | - | - | - | | | | | | | | ND | ND | ND | ND |
| | 62 | ND | - | - | - | | | | | | | | ND | ND | ND | ND |
| C | 48 | ND | - | - | - | -/+ | - | | | | | | - | - | - | - |
| o | 31 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| n | 37 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| t | 38 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| r | 39 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| o | 41 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| l | 42 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| | 45 | -/+ | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| | 50 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| | 59 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |
| | 63 | - | - | - | - | - | - | - | - | - | - | | - | - | - | - |

* liver biopsy performed 32 weeks after inoculation, **ICLN** ileocaecal lymph node, # clinical case, **ND** not done.

**PCR**: + presence of 400-bp band on a gel and a signal present on a dot blot; +/- signal present on a dot blot but no visible 400-bp band on a gel; - absence of 400-bp band on a gel and no signal on a dot blot.

**Histology:** + granulomatous lesions with AFO's (lepromatous morphology); +/- granulomatous lesions without AFO's (equivocal lesions of lepromatous morphology); (+/-) lesions showing central necrosis in which AFO's were not detected (equivocal lesions of tuberculoid morphology); - neither lepromatous- nor tuberculoid-like lesions detected (no AFO's).

**Figure 6.1.** Results of single PCR tests on selected samples of ileal and ileocaecal lymph node DNA, an equivalent of approximately 4 mg of tissue, from 14 sheep infected with *M. paratuberculosis*, 14 sheep vaccinated against paratuberculosis 2 weeks after infection and 11 uninfected-unvaccinated control sheep. Separation of 400-bp PCR products, generated by primers 90 and 9, on 2% gel (A, B and C). Dot blot results following hybridisation of the 400-bp PCR products with 194-bp DIG-labelled probe (D). Dots are labelled with the alphabetical code of the gel and numbers of gel's lanes. Samples in lanes A8, A9, A10, A23, B4, B15, B21, B22, C5, C6, C7, C12, C18, C20, C21 and C22 were classified as positive. Bands of 400-bp PCR products were present in these lanes and results of dot blot analysis were concordant. A sample in lane C19 was classified as suspicious since no band of 400-bp PCR products was visible on the gel but a hybridisation signal was obtained in the corresponding dot.

**Sheep ID and category:**

**c** control

**i** infected-unvaccinated

**v** infected-vaccinated

**Histology** (presence or absence of lesions consistent with paratubeculosis in the ileum):

**+** lesions with acid-fast organisms

**+/-** lesions without acid-fast organisms (equivocal)

**-** lesions not detected (no acid-fast organisms)

**PCR**:

**-ve** negative control (no DNA)

**+ve** positive control (*M. paratuberculosis* DNA)

**0** molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

**Figure 6.1.**

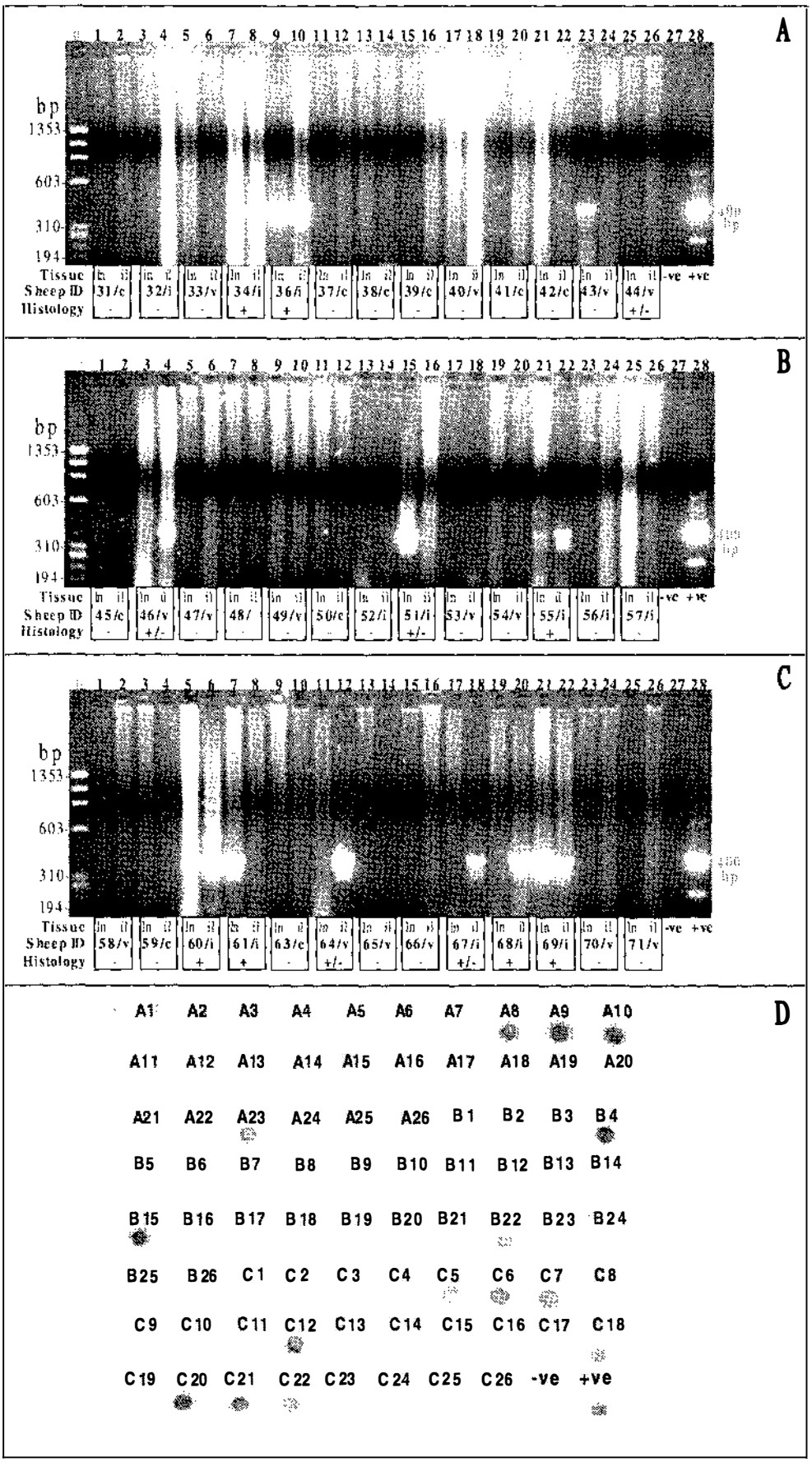

A
B
C
D
bp
1353
603
310
194
Tissue
Sheep ID
Histology
-ve
+ve
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10
A11 A12 A13 A14 A15 A16 A17 A18 A19 A20
A21 A22 A23 A24 A25 A26 B1 B2 B3 B4
B5 B6 B7 B8 B9 B10 B11 B12 B13 B14
B15 B16 B17 B18 B19 B20 B21 B22 B23 B24
B25 B26 C1 C2 C3 C4 C5 C6 C7 C8
C9 C10 C11 C12 C13 C14 C15 C16 C17 C18
C19 C20 C21 C22 C23 C24 C25 C26 -ve +ve

**Figure 6.2.** Results of single PCR tests on selected samples of hepatic (liver biopsy) DNA, an equivalent of approximately 4 mg of tissue, from 13 sheep infected with *M. paratuberculosis*, 14 sheep vaccinated against paratuberculosis 2 weeks after infection and 11 uninfected-unvaccinated control sheep. Liver biopsy specimens were collected 32 weeks after oral infection with *M. paratuberculosis*. Separation of 400-bp PCR products, generated by primers 90 and 91 in the Expand Long Template system, on 2% gel (A and B). Dot blot results following hybridisation of the 400-bp PCR products with 194-bp DIG-labelled probe (C). Dots are labelled with the alphabetical code of the gel and numbers of gel's lanes. Samples in lanes B10 and B18 were classified as positive. Bands of 400-bp PCR products were present in these 2 lanes and results of dot blot analysis were concordant. Samples in lanes A3 and A16 were classified as suspicious since no bands of 400-bp PCR products were visible on the gel but hybridisation signals were obtained in the corresponding dots.

**Sheep ID and category:**
**c** control
**i** infected-unvaccinated
**v** infected-vaccinated
**x** sheep that was not a part of this study

**Histology** (presence or absence of lesions consistent with paratubeculosis in the ileum):
**+** lesions with acid-fast organisms
**+/-** lesions without acid-fast organisms (equivocal)
**-** lesions not detected (no acid-fast organisms)

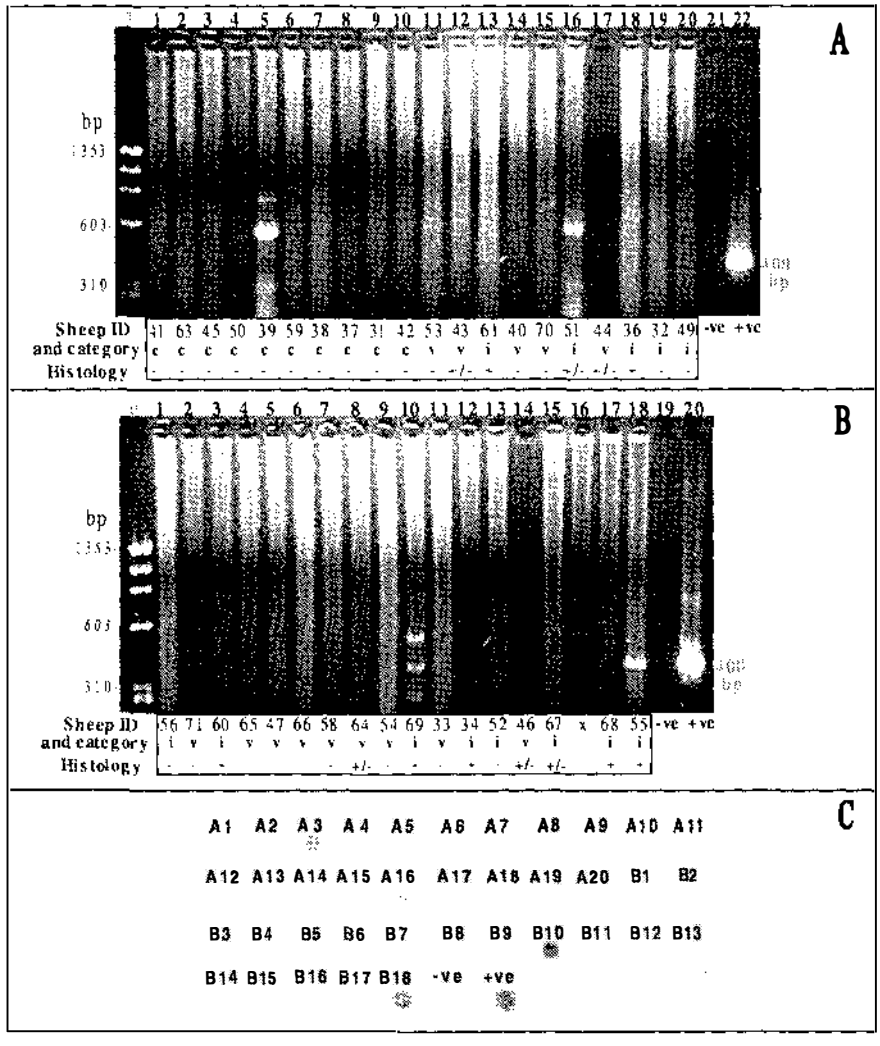
**PCR**:
**-ve** negative control (no DNA)
**+ve** positive control (*M. paratuberculosis* DNA
**0** molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

### 6.3.2. Liver biopsy PCR

The PCR on duplicate samples of DNA extracted from liver biopsy specimens was positive in 2 of 14 infected-unvaccinated sheep, both of which had histologically confirmed paratuberculosis at the time of necropsy. A suspicious reaction was obtained in one infected-unvaccinated sheep which had only equivocal lesions in the intestinal sections examined, but tested positive by the ileocaecal lymph node-based PCR. None of the vaccinated sheep had positive PCR results in the liver, although in 4 of them, equivocal lesions were later detected histologically in the ileum. One of the 11 control sheep was suspicious in the liver biopsy PCR. This sheep had no histological evidence of paratuberculosis and samples of ileum and ileocaecal lymph node from this animal also tested negative by the PCR at the time of necropsy.

The results of the PCR on selected liver biopsy specimens are presented in Figure 6.2.

**Figure 6.2.**



### 6.3.3. Blood PCR

In total, 340 blood samples were tested by the PCR over 53 weeks: 101 from uninfected-unvaccinated control sheep, 117 from infected-unvaccinated sheep and 122 from infected-vaccinated sheep. No positive or suspicious blood PCR results were detected in infected vaccinated and unvaccinated sheep within 18 weeks after oral inoculation with *M. paratuberculosis*. The blood-based PCR assay gave positive reactions in 2 individual samples from 2 of the 14 infected-unvaccinated sheep, both of which had histologically confirmed paratuberculosis at the time of necropsy, and in 3 individual samples from 3 of the 14 infected-vaccinated sheep. The 5 positive blood PCR results were obtained between the 18th and 27th week postinoculation. Among the 3 infected-vaccinated sheep identified as infected by the blood PCR, 2 had equivocal intestinal lesions, and the other had no lesions in any of the tissue sections examined. One of the 2 infected-vaccinated, blood PCR +ve sheep with equivocal intestinal lesions gave a positive PCR reaction in a sample of the ileocaecal lymph node. Of the 5 sheep identified as positive by the blood-based PCR, none of them showed a positive blood PCR reaction on more than one occasion, and only one was also identified as infected by the PCR on liver biopsy specimens.

Of the remaining 12 infected-unvaccinated and 11 infected-vaccinated animals, single samples from one unvaccinated and 3 vaccinated sheep gave suspicious results. The 4 suspicious blood PCR results were obtained between the 18th and 27th week postinoculation. The unvaccinated sheep that gave a suspicious blood PCR reaction had equivocal intestinal lesions but positive PCR results were obtained in samples of ileum and ileocaecal lymph node from this animal. Among the 3 vaccinated sheep that gave a suspicious blood PCR reactions, one had equivocal lesions in the ileocaecal lymph node and the remaining 2 had no histological evidence of paratuberculosis in tissues examined. Samples of ileum and ileocaecal lymph node from these 3 vaccinated sheep tested negative by the PCR.

One sample of blood from a control sheep gave a suspicious PCR reaction in the 9th week of the experiment. This animal died after 27 weeks of the study due to internal haemorrhage after hepatic rupture, and at that stage had no histological evidence of paratuberculosis. Furthermore, samples of ileum and ileocaecal lymph node from this

**Figure 6.3.** Results of the PCR on selected samples of peripheral blood leukocyte DNA, an equivalent of approximately 0.5 ml of blood, from 14 sheep infected with *M. paratuberculosis*, 14 sheep vaccinated against paratuberculosis 2 weeks after infection and 11 uninfected-unvaccinated control sheep. Samples of blood were collected 18 weeks after oral infection with *M. paratuberculosis*. Separation of 400-bp PCR products, generated by primers 90 and 91 on 2% gel (A and B). Dot blot results following hybridisation of the 400-bp PCR products with 194-bp DIG-labelled probe (C). Dots are labelled with the alphabetical code of the gel and numbers of gel's lanes. Samples in lanes A14, A16, A18 and B27 were classified as positive since bands of 400-bp PCR products were present in these lanes and results of dot blot analysis were concordant. The band in lane A14 is very weak, barely visible. The band in lane B27 is weak. Samples in lanes A21, B3 and B21 were classified as suspicious, as no bands of 400-bp PCR products were visible on the gels but hybridisation signals were obtained in the corresponding dots.

**Sheep ID and category:**

**c** control

**i** infected-unvaccinated

**v** infected-vaccinated

**x** sheep that were not a part of this study. Some of these sheep were experimentally infected with *M. paratuberculosis,* as described in Chapter 4. Two samples from these sheep, in lanes A19 and A24, were classified as positive.

**Histology** (presence or absence of lesions consistent with paratubeculosis in the ileum):

**+** lesions with acid-fast organisms

**+/-** lesions without acid-fast organisms (equivocal)

**-** lesions not detected (no acid-fast organisms)

**PCR**:

**-ve** negative control (no DNA)

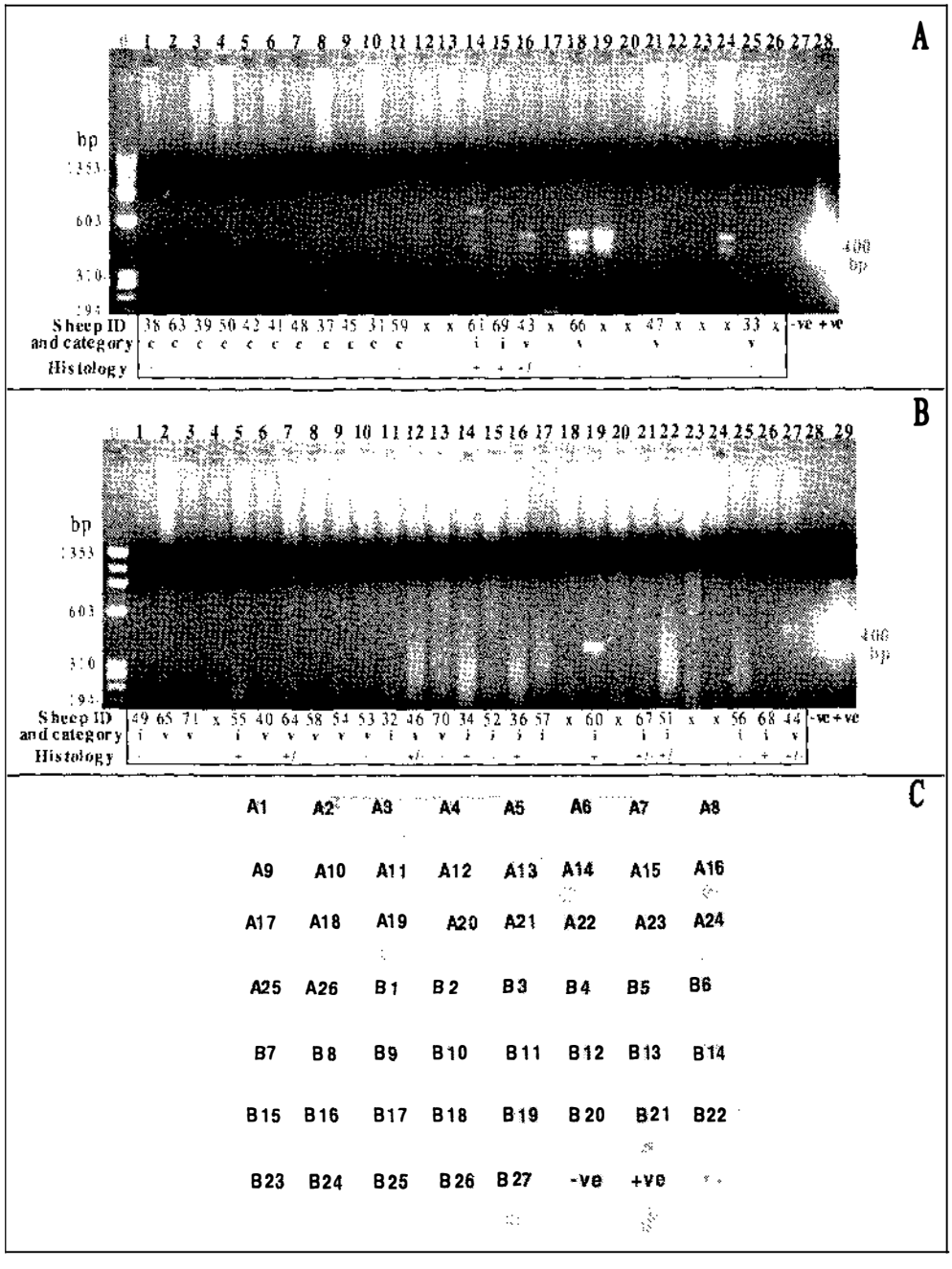
**+ve** positive control (*M. paratuberculosis* DNA)

**0** molecular size marker, ϕX174 RF DNA/*Hae* III Fragments

sheep tested negative by the PCR. Blood samples of the remaining 12 controls gave negative PCR results.

The results of the PCR on selected samples of blood are presented in Figure 6.3.

**Figure 6.3.**




## 6.4. Discussion

In the present study, the PCR assay gave positive reactions in only 2 of 117 blood samples that were sequentially collected, over 53 weeks, from 14 sheep inoculated

orally with *M. paratuberculosis* at the age of 1-1.5 months. The 2 positive results were obtained in 2 sheep that had typical granulomatous lesions containing acid-fast organisms in intestinal sections at the time of post-mortem examination. However, the assay failed to detect the target DNA in blood samples from the remaining 5 sheep with histologically confirmed paratuberculosis. Similarly, the PCR on liver biopsy specimens, collected 32 weeks after oral infection with *M. paratuberculosis*, gave only 2 positive results, both of which were obtained in 2 of the 7 sheep which had histologically confirmed paratuberculosis. One of the 2 sheep tested that positive by the liver biopsy-based PCR gave a positive reaction in the blood-based PCR.

Nine of the 14 experimentally infected sheep gave positive reactions in the PCR on samples of ileum and/or ileocaecal lymph node collected at the time of necropsy. If, instead of histology, the ileum- and ileocaecal lymph node-based PCR were used as the "gold standard", then 7 of 9 experimentally infected sheep would have not been diagnosed as infected by both the blood- and liver-based PCR. Unexpectedly, no positive PCR reaction was given by blood samples that were collected from experimental sheep around the time when these animals were showing clinical signs compatible with paratuberculosis. This is in contrast with the high detection rate of the target DNA in blood samples from naturally infected sheep with clinical paratuberculosis (see Chapter 3). The discrepancy may be due to a lower magnitude of extraintestinal infection in experimental animals as compared with the naturally infected sheep. Hepatic granulomas were detected in only 4 of 7 experimental sheep with clinical paratuberculosis, while all 12 naturally infected sheep had granulomatous lesions in the liver. The AFO's were detected in liver sections from one experimental animal and 5 naturally infected sheep. These results indicate that the PCR assay on blood and liver biopsy samples does not enhance detection of *M. paratuberculosis* in subclinical cases and clinically affected sheep with mild or moderate extraintestinal infection.

In addition to the 2 experimentally infected sheep that tested positive by the blood-based PCR, positive PCR reactions were obtained in 3 blood samples from 3 sheep which were vaccinated against paratuberculosis 2 weeks after oral inoculation with *M. paratuberculosis*. Interestingly, the 5 positive blood PCR results were obtained only between the 18th and 27th week postinoculation. This coincided with a peak of immune

response in the group of infected-vaccinated sheep and onset of detectable immunological reaction in the group of infected but unvaccinated sheep (see Chapter 5). The number of samples with positive results is too small to be conclusive, however, the possibility of a relationship between the presence of target DNA in blood and these early immunological events cannot be ruled out.

In the present study, there was no significant difference between the number of infected-vaccinated and infected-unvaccinated sheep tested positive by the PCR on samples of ileum and ileocaecal lymph node and the number of sheep identified as infected by histology. This suggests that the PCR assay used here offers little advantage over histology in the routine post-mortem diagnosis of paratuberculosis. The negative result of the lymph node-based PCR obtained in one infected-unvaccinated sheep that had scanty AFO's in the mesenteric lymph node probably represents a false negative reaction. Since the ileal sample of this sheep was PCR-positive, it is rather unlikely that infection in the lymph node was caused by a different mycobacterial species. The possible explanations for this negative result include inadequate extraction of *M. paratuberculosis* from tissues, insufficient extraction of DNA from *M. paratuberculosis*, the presence of PCR inhibitors, or a combination of these.

The failure to consistently detect *M. paratuberculosis* DNA in liver biopsy and blood samples may reflect intermittent presence or absence of the target DNA in these tissues. Alternatively, it might have been due to either inadequate sensitivity of the PCR assay or insufficient volume of samples. In human medicine, 30 ml of blood is the optimal sample volume for bacteriological culture (Reimer, 1997). In this study, 0.5 ml of blood was tested by the PCR assay. However, due to inhibition of the amplification process by extraneous substances present in diagnostic specimens (Lo *et al.*, 1989; Mercier *et al.*, 1990; Rossen *et al.*, 1992; Lo, 1994), testing of large-volume samples may be counterproductive. The results of this study seem to further exemplify the limitations of this new technology in the diagnosis of infectious diseases. Nevertheless, the rapid availability of PCR results is the major advantage of this technique over the culture method. Therefore, future attempts to increase sensitivity of the PCR should be focused on the improvement of methods of recovery and purification of target DNA from clinical samples. The recently reported hybridisation-capture PCR method (Millar *et al.*, 1995) offers some prospects for such improvement. The combination of the BACTEC

radiometric culture system and the PCR-based identification of the mycobacterial growth has also been reported to be both more sensitive and quicker than the routine culture (Cousins *et al.,* 1995; Whittington *et al.,* 1998).

In the present study, samples were classified as suspicious when no band of the right size (400 base pair) was visible on the gel but a signal was present on the dot blot. The interpretation of these suspicious PCR results is difficult. As the dot blot analysis is more sensitive than visual examination of a gel, such reactions obtained in the experimentally infected sheep may represent the detection of small amounts of PCR products that were indiscernible on a gel. However, the samples contained large amounts of mammalian DNA and the dot blot signals, frequently very weak, could also be due to non-specific binding of the probe to the mammalian DNA. Similarly, the 2 suspicious results obtained in 2 control sheep, one in a blood sample and one in a liver biopsy specimen, may represent either non-specific cross-reactivity detectable by hybridisation or the detection of small amounts of PCR products. If these suspicious reactions were due to the presence of PCR products in the samples, then it could indicate either contamination of samples or detection of infection. The latter is possible, as the control animals were exposed to sheep of unknown infection status, but seems unlikely in the light of poor discriminatory powers of the PCR in subclinically infected sheep.

In summary, under conditions of this study the PCR assay on samples of blood and liver biopsy specimens does not enhance detection of *M. paratuberculosis* in subclinically infected sheep and clinically affected sheep with mild or moderate extraintestinal infection. Furthermore, considering the need for laborious preparation of samples, it appears that the PCR assay on samples of ileum and ileocaecal lymph node offers little advantage over histology in a routine post-mortem diagnosis of paratuberculosis.

# Chapter 7

# Comparison of a complement fixation test, gel immunodiffusion test, enzyme-linked immunosorbent assay and interferon-γ assay for the diagnosis of paratuberculosis in sheep infected experimentally with *Mycobacterium paratuberculosis*

## 7.1. Introduction

The use of serology in the diagnosis of paratuberculosis is considered to be of limited value (Kreeger, 1991). The principal reason for this is that seroconversion occurs relatively late in the course of infection (Chiodini *et al.*, 1984b; Cocito *et al.*, 1994). Among the available tests, the complement fixation test (CFT), agar gel immunodiffusion test (AGID) and enzyme-linked immunosorbent assay (ELISA) have recently been reported to have similar discriminatory powers for detecting sheep with clinical paratuberculosis, while the AGID and ELISA seem to be more sensitive in identifying subclinically infected sheep than is the CFT (Hilbink *et al.*, 1994).

The detection of cell-mediated immune (CMI) response has been reported to precede detectable antibody production in cattle infected orally with *Mycobacterium paratuberculosis* (Lepper *et al.*, 1989). In the past, the degree of delayed-type hypersensitivity after intradermal injection of *M. paratuberculosis* antigen was used to measure a CMI response. This intradermal test is considered not useful for the diagnosis of paratuberculosis in sheep (Quinn *et al.*, 1994), while in cattle it lacks accuracy (Larsen *et al.*, 1963; Merkal *et al.*, 1968b; de Lisle *et al.*, 1980a). The interferon-γ assay (IFN-γ), an alternative method to measure CMI response, has been recently evaluated for the diagnosis of paratuberculosis in cattle (Billman-Jacobe *et al.*, 1992; Stabel, 1996). The results of both these studies indicate that this test may be an important tool for the detection of cows subclinically infected with *M. paratuberculosis*.

Two components of diagnostic performance of a serological test, sensitivity and specificity, are usually estimated against results obtained by histology or culture of the organism. Sensitivity of a test depends on the concentration of antibodies required to give a positive result and subsequently it is affected by the kinetics of the humoral response. Accurate determination of the sensitivity of tests for paratuberculosis is difficult due to the slow progressive character of the disease and limitations of the culture and histology in their ability to accurately diagnose early infection.

The experimental models, described in Chapters 4 and 5, created an opportunity to study the effect of antibody kinetics on the diagnostic performance of three commercially available serological tests for paratuberculosis: the CFT, AGID and ELISA in a population of sheep with a known infection status. In addition, the potential suitability of the IFN-γ assay for the diagnosis of paratuberculosis in sheep was assessed.

## 7.2. Materials and methods

### 7.2.1. Animals and collection of samples for immunological testing

The source of experimental sheep, the husbandry conditions under which they were maintained, the dose of *M. paratuberculosis* used to infect the sheep orally, along with the collection schedule of samples of blood for serological testing and IFN-γ assay have been described in Chapter 4 and 5 and will be only summarised briefly in this chapter.

#### 7.2.1.1. Trial 1

In the first trial (described in Chapter 4), 30 newborn lambs, and their mothers, were obtained from a flock where paratuberculosis had not previously been diagnosed. The lambs were inoculated orally, initially at the age of 1 week and a second time 2-3 weeks later, with approximately $3.4 \times 10^9$ *M. paratuberculosis* organisms. The lambs were weaned at the age of 7-9 weeks. Between the 19th and 75th week after oral inoculation with *M. paratuberculosis*, 20 of the 30 sheep developed clinical signs

compatible with paratuberculosis and either died (3 sheep) or were sacrificed by intravenous injection of pentobarbitone. The experiment was terminated 108 weeks postinoculation when the remaining 10 experimentally infected sheep were killed by intravenous injection of pentobarbitone.

Samples of blood for serological tests were collected from the jugular vein of each sheep into 4 ml plain evacuated tubes, initially 10 weeks postinoculation and then 19, 23, 27, 36, 40, 45, 49, 53, 64, 74,82, 91, 100 and 108 weeks after inoculation.

### 7.2.1.2. Trial 2

In the second trial (described in Chapter 5), 28 of 41 lambs were inoculated once via a stomach tube with approximately $4.4 \times 10^8$ *M. paratuberculosis* organisms at the age of 1.0-1.5 months. Two weeks later, 14 of the 28 experimentally infected lambs were vaccinated by subcutaneous injection of 1 ml of live-attenuated vaccine (Rhone Merieux, France). The infected-vaccinated group was excluded from this study. The remaining 13 uninfected lambs were used as negative controls. Three of the 13 control sheep died for causes unrelated to paratuberculosis (see Appendix B.4). Between the 32nd and 53rd week after oral inoculation, 7 of the 14 experimentally infected animals developed clinical paratuberculosis. All of these clinical cases were sacrificed by intravenous injection of pentobarbitone. The experiment was terminated 53 weeks postinoculation when the remaining 7 experimentally infected sheep and 10 controls were killed by intravenous injection of pentobarbitone.

Blood was collected from the jugular vein of each sheep into 4 ml plain evacuated tubes for serological tests and into 4 ml vacutainer tubes containing lithium heparin for the interferon-γ (IFN-γ) assay. In this trial, the blood samples were collected initially on the day of oral inoculation with *M. paratuberculosis* and then 2, 5, 9, 18, 27, 36, 44 and 53 weeks after inoculation. As all 41 lambs were obtained from a flock where paratuberculosis had not previously been diagnosed, samples collected from these lambs at the time of oral inoculation were included into validation of specificity of immunological tests.

### 7.2.2. Animals infection status

The infection status of 28 sheep infected experimentally with *M. paratuberculosis* in Trial 1, 14 sheep infected experimentally with *M. paratuberculosis* in Trial 2 and 11 uninfected control sheep was determined by histology and a PCR assay on samples of ileum and ileocaecal lymph node at the time of necropsy as described in Chapters 4 and 5. Samples of tissues from 2 sheep infected experimentally with *M. paratuberculosis* in Trial 1, and from 2 control sheep that died in the early stage of the second trial (see Appendix B.4), were not available for postmortem examinations.

### 7.2.3. Sample processing and immunological tests

Processing of blood samples and testing of serum samples in serological tests (CFT, AGID and ELISA) and plasma samples in the IFN-γ assay have been described in detail in Chapters 4 and 5 and will be only summarised briefly here.

***Serological tests***

The CFT and AGID test were performed at The Central Animal Health Laboratory, Upper Hutt, New Zealand using the method described by Hilbink *et al.* (1994).

The ELISA assay (Paracheck™, CSL, Australia) was performed and its results validated according to the manufacturer's recommendations as described in Chapters 4 and 5 (see Appendix A.2 for details). Because this assay was developed for detection of bovine antibodies, the cut-off value of 0.1 plus the mean optical density value (OD) of negative controls, as recommended by the manufacturer, and a low cut-off value of 0.05 were used to interpret the results. A result was considered positive when the mean OD of test samples was equal to or greater than the predetermined cut-off.

***IFN-γ assay***

Samples of heparinised blood were processed, and the IFN-γ assay (Bovigam™, CSL, Australia) was performed and its results validated, as described in Chapter 5 (see Appendix B.2 for details). The cut-off value of 0.05, as recommended by the manufacturer, was used to interpret the results of the IFN-γ assay. The mean OD of nil antigen (PBS) and Johnin-stimulated samples of each animal were compared. A result

was considered positive when the difference between the mean OD of Johnin-stimulated samples and mean OD of nil antigen (PBS) samples was equal to or greater than the predetermined cut-off.

### 7.2.3. Statistical analysis

A sheep was considered as detected (identified as infected) by an immunological test, if any of the samples that were collected sequentially from this animal gave at least one positive reaction in this test. In order to facilitate comparison of immunological assays, detection curves, i.e. the cumulative number of sheep detected by an assay over time, were generated. For generation of the detection curves, only sheep detected by an immunological test for the first time were counted. The detection curves of immunological assays were compared using the Logrank test. The Fisher exact test was used to analyse data presented in Tables C.1 and C.2 in Appendix C.

All statistical analyses were performed using the GraphPad Prism, version 2.01, program (GraphPad Software Incorporated, USA).

## 7.3. Results

### 7.3.1. Trial 1

The results of the CFT, AGID, ELISA with low 0.05 cut-off (0.05-ELISA) and ELISA with high 0.1 cut-off (0.1-ELISA) in 30 sheep inoculated orally with *M. paratuberculosis* in the first trial are presented in Figure 7.1. The seroresponse in these sheep was frequently intermittent, or transient, and the serological tests often showed a poor agreement in results. Among the serological test, only the 0.05-ELISA detected all 14 experimentally infected sheep which had paratuberculosis confirmed by histology and PCR. The serological tests, except the 0.1-ELISA, showed poor discrimination between the 14 sheep that had paratuberculosis subsequently confirmed by histology and PCR, and the 9 experimentally infected sheep that tested negative by PCR and had no histological evidence of paratuberculosis, 108 weeks postinfection (see Appendix C.1; Table C.1). The highest number of positive results in the serologi-

**Figure 7.1.** Results of the CFT, AGID, ELISA 0.1 cut-off and ELISA 0.05 cut-off in samples of serum sequentially collected from 30 sheep inoculated orally with *M. paratuberculosis* in the first month of life (Trial 1).

**Histology results** (presence or absence of lesions consistent with paratuberculosis in the ileum):

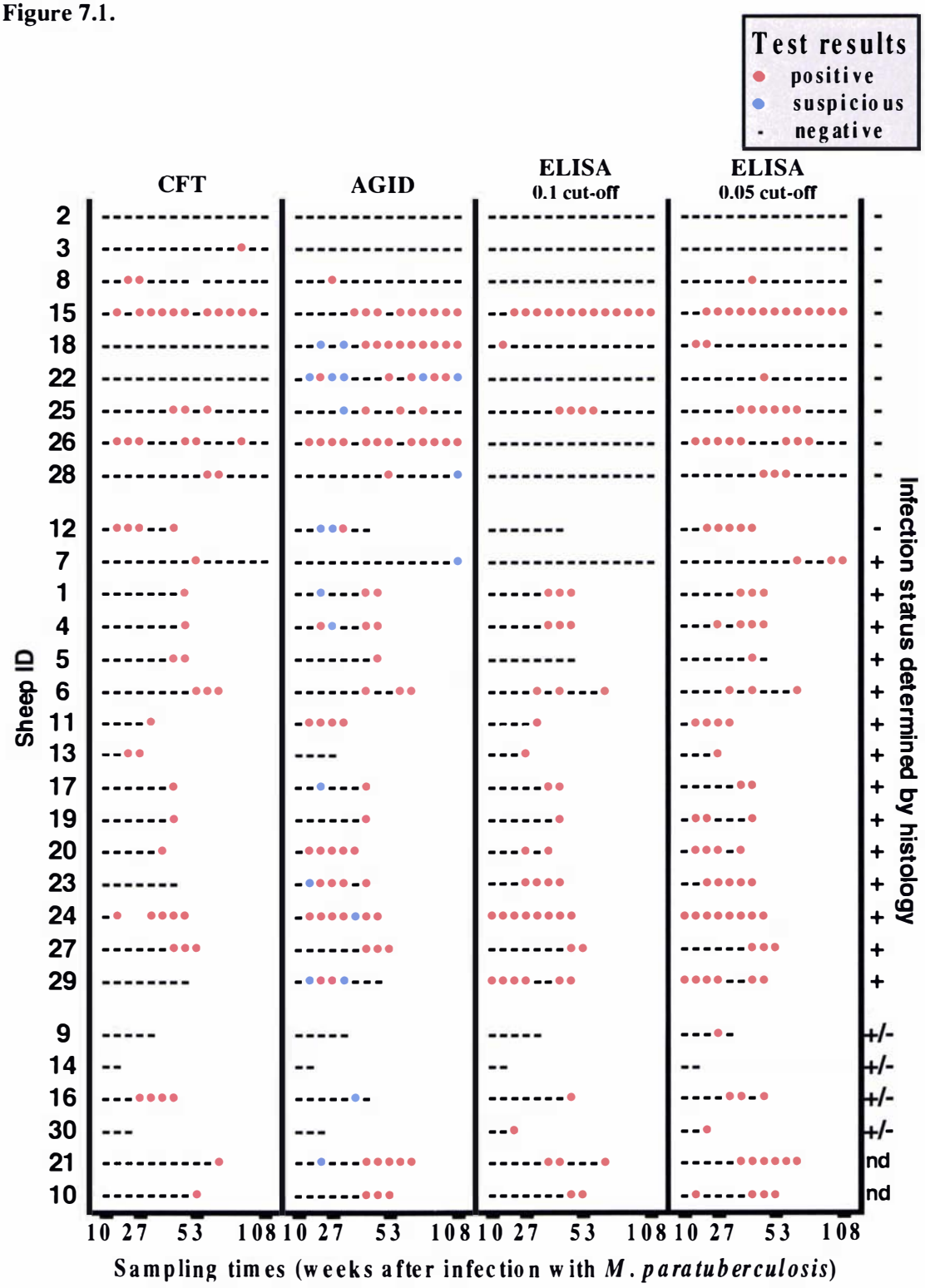
+ lesions with acid-fast organisms

+/- lesions without acid-fast organisms (equivocal)

- lesions not detected (no acid-fast organisms)

cal tests was observed during winter, between the 27th and 53rd week after inoculation (see Appendix C.1)

**Figure 7.1.**

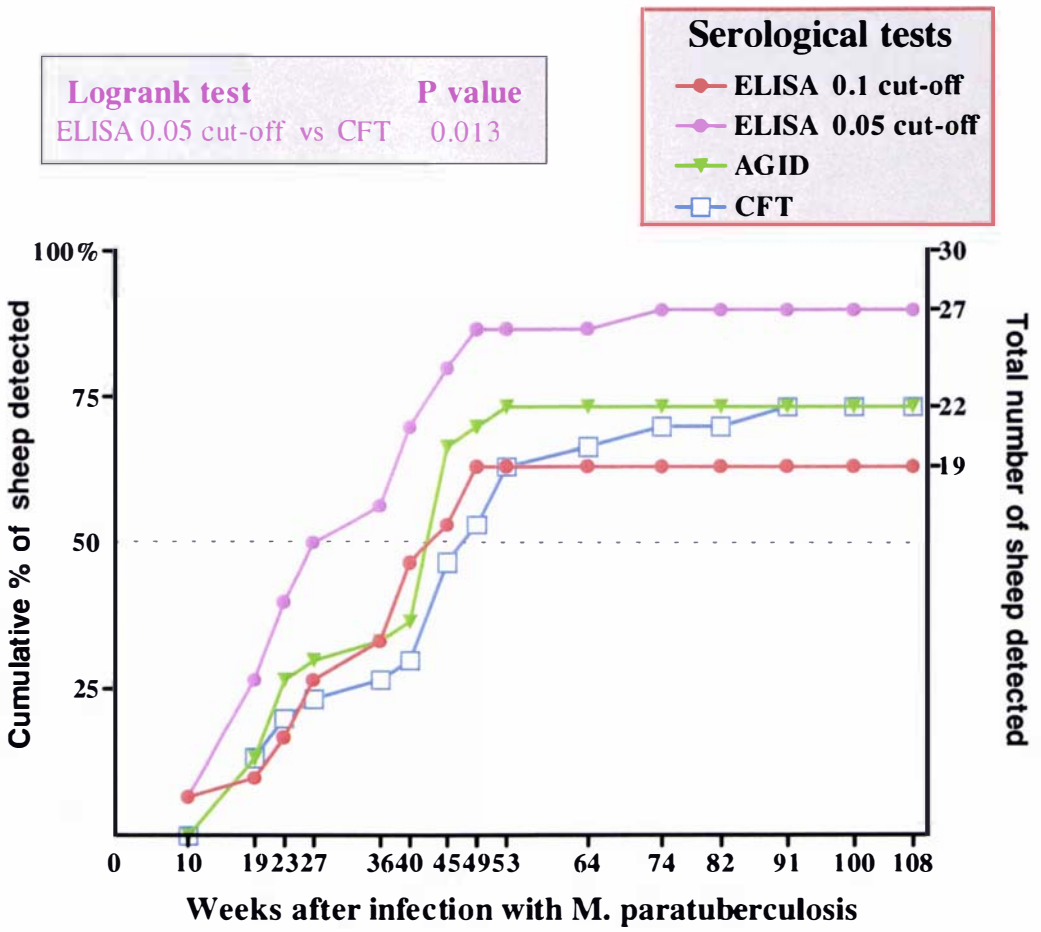



The detection curves of serological tests used in Trial 1 are presented in Figure 7.2. The detection rates of the serological tests (percentages of sheep detected) were as follows:

**Figure 7.2.** Detection curves of the CFT, AGID, ELISA 0.1 cut-off and ELISA 0.05 cut-off in a group of 30 sheep inoculated orally with *M. paratuberculosis* in the first month of life (Trial 1). The detection curves (cumulative numbers of animals detected over time) were compared using the Logrank test. A sheep was considered to be detected (identified as infected) by an immunological test if any of the samples from this animal gave at least one positive reaction in this test. For generation of the detection curves, only sheep detected by an immunological test for the first time were counted.

63% for the 0.1-ELISA, 73% for the CFT and AGID and 90% for the 0.05-ELISA. The median detection times (time after infection by which at least 50% of sheep were detected by a serological test) were the 27th week for the 0.05-ELISA, 40th week for the 0.1-ELISA, 45th week for the AGID and 49th week for the CFT. During the first trial the 0.05-ELISA detected significantly (P=0.0126) more experimentally infected sheep, and over shorter period, than the CFT.

**Figure 7.2.**




### 7.3.2. Trial 2

The results of the IFN-γ assay, CFT, AGID, ELISA with low 0.05 cut-off (0.05-ELISA) and ELISA with high 0.1 cut-off (0.1-ELISA) in 14 sheep inoculated orally with *M. paratuberculosis* in the second trial are presented in Figure 7.3. As in the first trial, the seroresponse in these sheep was frequently intermittent or transient and the sero-

**Figure 7.3.** Results of the CFT, AGID, ELISA with 0.1 cut-off, ELISA with 0.05 cut-off and IFN-γ assay in serum and plasma samples sequentially collected from 14 sheep inoculated orally with *M. paratuberculosis* in the second month of life (Trial 2).

**Histology results** (presence or absence of lesions consistent with paratuberculosis in the ileum):

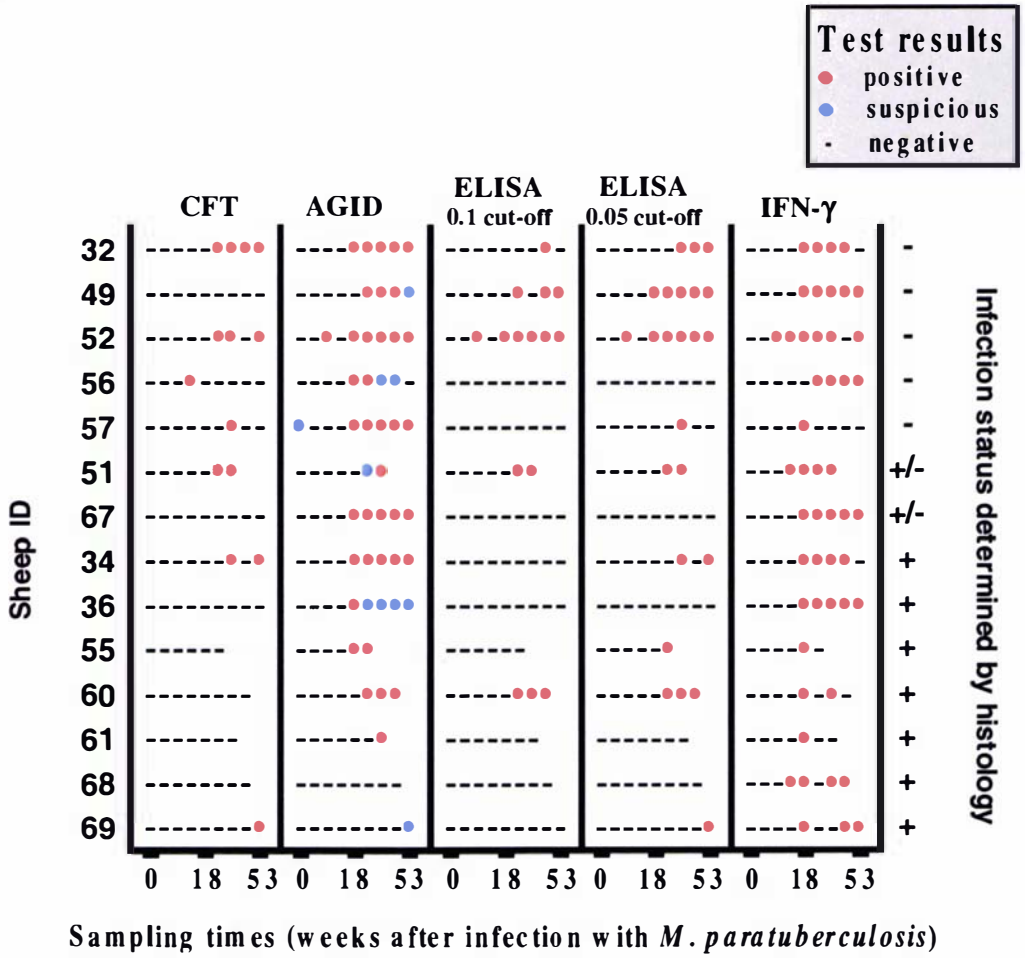
+ lesions with acid-fast organisms

+/- lesions without acid-fast organisms (equivocal)

- lesions not detected (no acid-fast organisms)

logical tests often showed a poor agreement in results. Likewise, the cell-mediated immune response, measured by the IFN-γ assay, was frequently transient or intermittent. Among the immunological tests, only the IFN-γ assay detected all 7 experimentally infected sheep which had paratuberculosis confirmed by histology and PCR. The immunological tests showed poor discrimination between the 7 sheep that had paratuberculosis later confirmed by histology and PCR, and the 5 experimentally infected sheep that tested negative by PCR and had no histological evidence of paratuberculosis at the time of necropsy, 53 weeks postinfection (see Appendix C.2; Table C.2). Similarly to the first trial, the highest number of positive results in the immunological tests was observed during winter, between the 27th and 53rd week after inoculation (see Appendix C.2).

**Figure 7.3.**

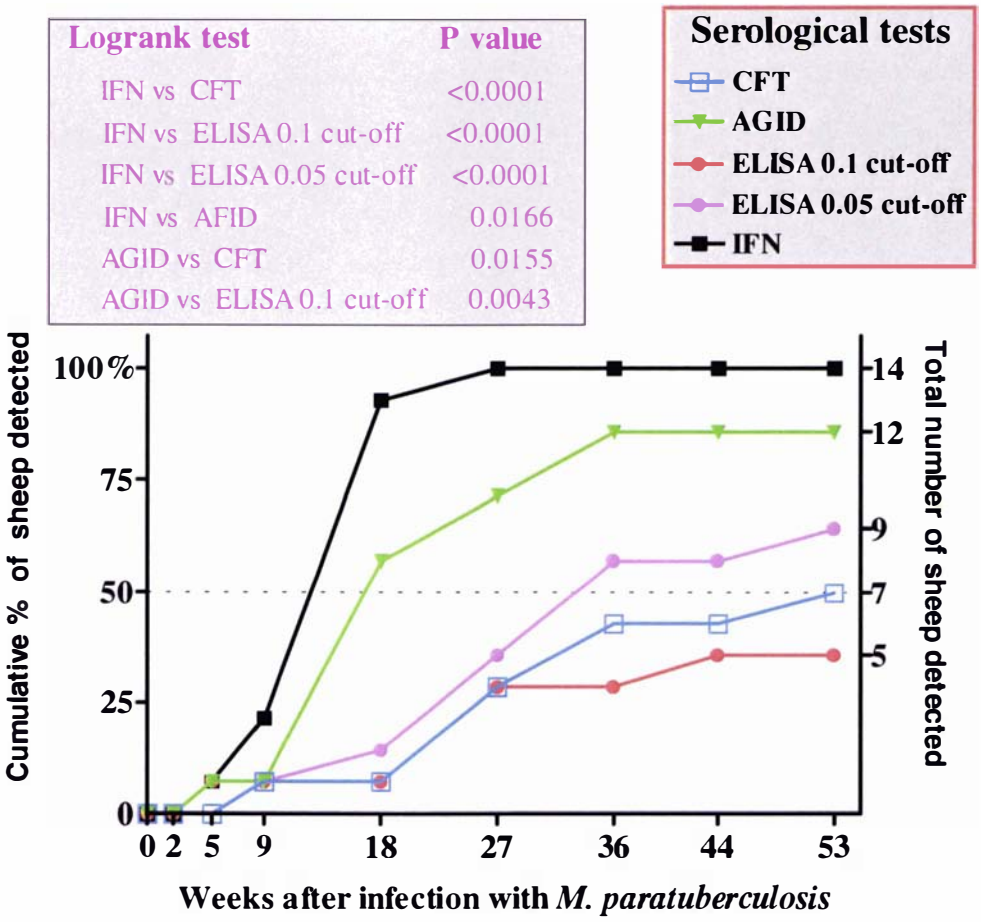



The detection curves of immunological tests used in Trial 2 are presented in Figure 7.4. The detection rates of immunological tests were as follows: 36% for the 0.1-ELISA,

**Figure 7.4.** Detection curves of the CFT, AGID, ELISA 0.1 cut-off, ELISA 0.05 cut-off and IFN-γ assay in a group of 14 sheep inoculated orally with *M. paratuberculosis* in the second month of life (Trial 2). The detection curves (cumulative numbers of animals detected over time) were compared using the Logrank test. A sheep was considered to be detected (identified as infected) by an immunological test if any of the samples from this animal gave at least one positive reaction in this test. For generation of the detection curves, only sheep detected by an immunological test for the first time were counted.

50% for the CFT, 64% for the 0.05-ELISA, 86% for the AGID and 100% for the IFN-γ assay. Both the IFN-γ assay and AGID gave positive reactions in at least 50% of sheep by the 18th week after infection. The median detection times of the 0.05-ELISA and CFT were the 36th and 53rd week postinfection, respectively. As presented in Figure 7.4, the diagnostic performance, i.e. cumulative numbers of sheep detected over time, varied significantly between tests.

**Figure 7.4.**




### 7.3.3. Validation of specificity of immunological tests

The results of the IFN-γ assay, CFT, AGID, ELISA with low 0.05 cut-off (0.05-ELISA) and ELISA with high 0.1 cut-off (0.1-ELISA) in sequentially collected samples of serum from 13 negative control sheep are presented in Figure 7.5. None of the serum samples was positive in the 0.1-ELISA and in the AGID test. Four samples from 4 control sheep gave suspicious reactions in the AGID. One sample from one sheep tested positive by the IFN-γ assay, 2 samples from 2 animals were positive in the CFT. The

**Figure 7.5.** Results of the CFT, AGID, ELISA with 0.1 cut-off, ELISA with 0.05 cut-off and IFN-γ assay in serum and plasma samples sequentially collected from 13 uninfected control sheep.

**Histology results** (presence or absence of lesions consistent with paratuberculosis in the ileum):

+ lesions with acid-fast organisms

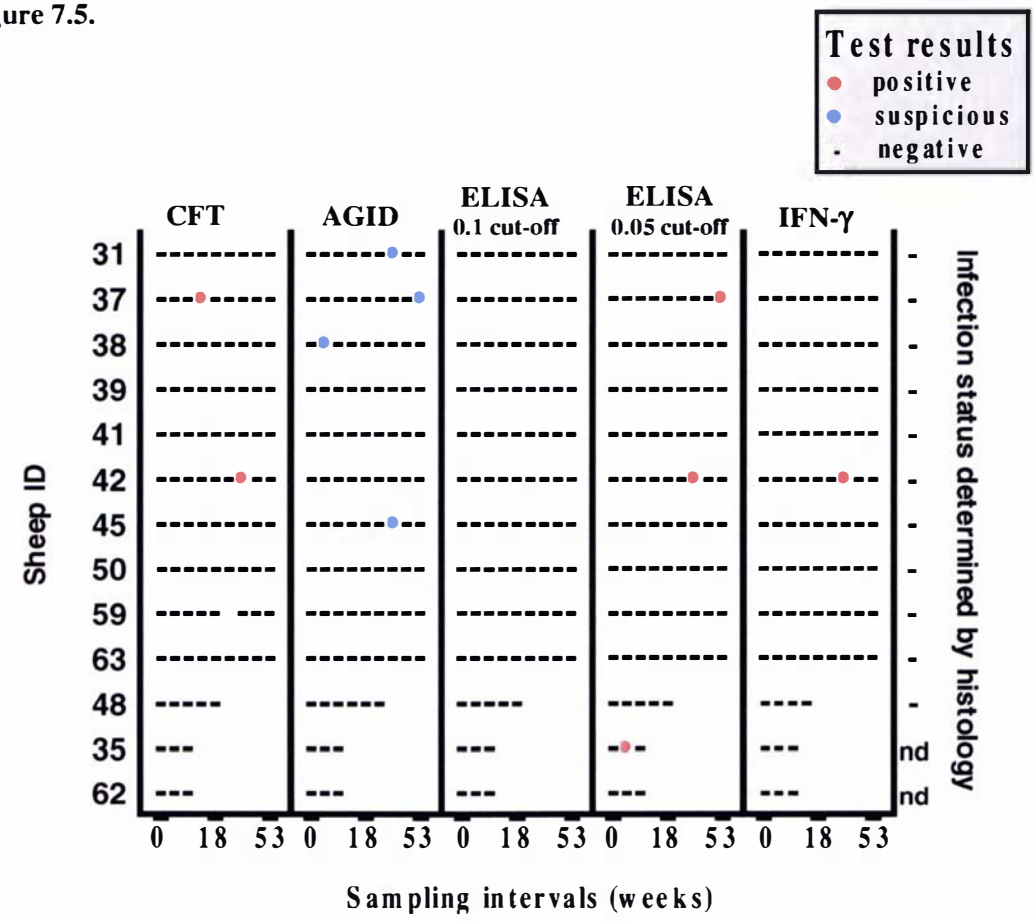
+/- lesions without acid-fast organisms (equivocal)

- lesions not detected (no acid-fast organisms)

* specificities calculated on results obtained in 41 samples of plasma and serum that were collected at the time of oral infection with *M. paratuberculosis* from 28 experimentally infected sheep, 14 of which were vaccinated against paratuberculosis 2 weeks after infection, and 13 uninfected and unvaccinated (control) sheep.

0.05-ELISA gave 3 positive reactions in 3 samples from 3 control sheep, one of which was obtained in an animal with undetermined infection status. This animal died early in the experiment and was not available for postmortem examinations.

**Figure 7.5.**




Specificities of the CFT, AGID, ELISA 0.1 cut-off, ELISA 0.05 cut-off and IFN-γ assay, calculated at all sampling points, are presented in Table 7.1. Suspicious results obtained by the AGID were excluded from the calculations.

**Table 7.1. Specificities of immunological tests at various sampling points.**

| Test | Calculated specificity (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sampling times / weeks | | | | | | | | |
| | 0* | 2 | 5 | 9 | 18 | 27 | 36 | 44 | 53 |
| CFT | 100* | 100 | 100 | 91 | 100 | 100 | 90 | 100 | 100 |
| AGID | 100* | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| ELISA 0.1 | 100* | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| ELISA 0.05 | 100* | 92 | 100 | 100 | 100 | 100 | 90 | 100 | 90 |
| IFN -γ | 100* | 100 | 100 | 100 | 100 | 100 | 90 | 100 | 100 |

## 7.4. Discussion

In the two longitudinal studies presented here, none of the serological tests was able to detect all sheep that were infected experimentally with *M. paratuberculosis*. In contrast, the IFN-γ assay, which was assessed only during the second trial, detected all 14 infected sheep by the 27th week after infection. This assay detected significantly more experimentally infected sheep, and over shorter period, than any of the serological tests. Furthermore, its specificity was high and similar to that achieved by the antibody assays. Although the number of animals used in this study is too small to be definitive, these results warrant further investigation of the use of the IFN-γ assay for the detection of sheep infected with *M. paratuberculosis*. This is supported by the fact that data obtained in cattle also indicate the potential usefulness of this test in the diagnosis of animals with subclinical paratuberculosis (Billman-Jacobe *et al.,* 1992; Stabel, 1996).

The failure of serological tests to detect all experimentally infected sheep might have been due to a number of factors. Apart from the intermittent presence of antibodies at the concentration required to give a positive result, it may be attributed to low sensitivity of the tests. In addition, it may be due to inappropriate time of sample collection or low magnitude of humoral response in some sheep. The possibility that oral inoculation with *M. paratuberculosis* was unsuccessful in some sheep also cannot be ruled out, however, this seems rather unlikely since all sheep were inoculated via a stomach tube.

In the past, the AGID was usually recommended as the most efficient test for sheep (Julian, 1975; de Lisle and Wall, 1983). Furthermore, this antibody assay has recently been reported to be the most specific test, with reasonable sensitivity, for identifying sheep in the later stages of the disease (Dubash *et al.,* 1996). The consistent and relatively high detection rates of the AGID during both trials (73% in the first trial and 86% in the second trial), along with relatively short median detection times (45th and 18th week after inoculation during the first and second trial, respectively) support these recommendations. The CFT detection rate ranged from 73% in the first trial to 50% in the second trial and its median detection times were 49 and 53 weeks after inoculation, respectively. The prolonged median detection times indicate that this test detects sheep in the more advanced stage of infection. This is in agreement with previous data (Hilbink *et al.,* 1994), and suggests that the CFT is unlikely to be suitable for detecting

subclinically infected sheep. The performance of the ELISA assay was inconsistent and its detection rates at the recommended 0.1 cut-off value, 63% in the first trial and 36% in the second trial, were lower than those achieved by the AGID and CFT. This assay is intended for the detection of bovine antibodies and better accuracy might be expected with a test developed specifically for sheep. However, since seroconversion occurs late in the course of infection, such an assay may not have a great impact on the diagnosis of ovine paratuberculosis. Alternatively, the use of lower than the recommended cut-off might be considered. When a low 0.05 cut-off was used to interpret the ELISA results, the detection rates were 90% in the first trial and 64% in the second trial. This increase in sensitivity was associated with a slight loss of the assay's specificity. Nevertheless, specificities of all immunological assays were similar and ranged from 90 to 100% at various sampling points. Occasional positive or suspicious reactions obtained by the AGID, ELISA with low 0.05 cut-off, CFT and IFN-γ assay in samples from the control sheep may reflect cross-reactivity to organisms sharing common epitopes with *M. paratuberculosis*. Alternatively, they might have been due to the exposure to the organism, since occasionally, the control sheep shared grazing with sheep of unknown infection status. Despite the methodological inadequacy, the low number of the positive and suspicious reactions suggests that the immunological tests are highly specific.

Generally, none of the antibody assays used in the current study was able to detect all sheep with histologically confirmed paratuberculosis. Their inability to identify animals that pose the greatest risk of spreading the infection further supports the general opinion that serological tests have limited value for the control of ovine paratuberculosis (Hilbink *et al.*, 1994).

Both the antibody assays and IFN-γ assay showed poor discrimination between experimentally infected sheep that had paratuberculosis confirmed by histology and PCR, and those that tested negative by PCR and had no histological evidence of paratuberculosis. This indicates that a positive reaction in any of the immunological assays could only be considered as an indication of exposure to *M. paratuberculosis*.

The parameters used in this study to assess diagnostic performance of the ELISA, CFT and AGID, such as detection rate and median detection time, varied between the first and second trial. The discrepancies are most likely due to differences in the study de-

sign and sampling schedule. In the first trial, the experimental sheep were inoculated twice, initially at the age of 1 week and a second time 2-3 weeks later, with approximately $3.4x10^9$ *M. paratuberculosis* organisms. In the second trial, the experimental sheep were inoculated only once with approximately $4.4x10^8$ *M. paratuberculosis* organisms at the age of 1-1.5 months. In the first trial, the infective material was obtained from 3 sheep with naturally acquired paratuberculosis, whereas the inoculum used in the second trial was derived from both naturally and experimentally infected sheep. The CFT and AGID test were performed sequentially throughout the duration of both trials and slight methodological differences might have also contributed to the discrepancies observed. Since the ELISA assay was performed after termination of both trials, and samples of serum were tested under similar conditions, it is less likely that the methodology had affected the results of this assay.

In both experiments presented here, the highest number of seropositive results in sheep infected experimentally with *M. paratuberculosis* was observed during the end of first winter after inoculation. This coincided with harsh climatic and nutritional conditions, and may be related to effect of stress on the immune system, allowing infection to become active. If, a similar pattern of seroresponse occurs in natural infection then the effectiveness of control or accreditation programs based on serological diagnosis of paratuberculosis may be affected by the time of testing. Further field trials are required to establish, or rule out, the potential relationship between the time of a year and course of humoral response in animals naturally infected with *M. paratuberculosis*.

In summary, the IFN-γ assay had high specificity and was shown to be a sensitive and early indicator of exposure to *M. paratuberculosis* in experimentally infected animals. This indicates the potential utility of this assay for detection of sheep infected with the organism. However, further studies are required to optimise the test with respect to the incubation time, amount and type of antigen(s) used in cell stimulation and volume and type of samples (whole blood versus peripheral blood mononuclear cells). There is also a need for both *M. paratuberculosis*-specific antigen(s) and non-specific T-cell stimulators. The latter are required to assess cell viability and identify false-negative reactions.

Among the serological tests used, the AGID had a reasonable and consistent detection

rate of sheep inoculated with *M. paratuberculosis* in both experiments. The ELISA assay showed an inconsistent diagnostic performance during the first and second experiment. This assay was developed for cattle and better accuracy might be expected with a test developed for sheep. The CFT appears to be less suitable for diagnosis of ovine paratuberculosis. However, the overall inability of serological tests to detect all sheep with histologically confirmed paratuberculosis supports the general opinion that serological tests have limited value in the diagnosis of subclinical ovine paratuberculosis.

# Chapter 8

# Antigen-induced production of interferon-γ in samples of peripheral lymph nodes from sheep infected experimentally with *Mycobacterium paratuberculosis*

## 8.1. Introduction

Interferon-γ (IFN-γ), a cytokine produced predominantly by T lymphocytes and NK cells, is crucial for activation of macrophages (Young and Hardy, 1995). Its role in the immunity to mycobacteria in mice is well documented (Cooper *et al.,* 1993; Kamijo *et al.,* 1993; Flesch *et al.,* 1995). The regulation of expression of the IFN-γ gene is complex. Vitamin $D_3$ [$1,25(OH_2)D_3$] (Reichel *et al.,* 1987; Daynes *et al.,* 1996) and glucocorticoids (Doherty *et al.,* 1995; Goff 1996) have been reported to suppress IFN-γ production. It has also been noted that pregnancy causes decreased antigen-specific IFN-γ response in lymphoid tissues of mice infected with *Leishmania major* (Krishnan *et al.,* 1996). Studies with human lymphocytes have shown that memory T cells are potent producers of this cytokine, while naive T cells have impaired ability to produce IFN-γ (Wilson *et al.,* 1986; Sanders *et al.,* 1988). In mice and cattle, the competence to produce IFN-γ following *in vitro* challenge with antigen varies between subsets of T cells (Mosman *et al.,* 1986; Bassey and Collins, 1997).

The measurement of IFN-γ production by peripheral blood mononuclear cells has recently been reported to be a useful diagnostic tool for the detection of cows subclinically infected with paratuberculosis (Billman-Jacobe *et al.,* 1992; Stabel 1996). As described in the previous chapter, in the blood of some sheep infected experimentally with *M. paratuberculosis*, the antigen-specific IFN-γ response could be detected above the predetermined cut-off level only during a relatively short period of time after exposure to the organism. Premier *et al.* (1996) have reported that in sheep antigen-specific memory T cells migrate preferentially to peripheral lymph nodes, re-

gardless of the route of immunisation.

The purpose of the study presented in this chapter was to determine whether antigen-specific production of IFN-γ above the commonly used cut-off level could be detected in samples of prescapular lymph node from sheep infected experimentally with *M. paratuberculosis*.

## 8.2. Materials and methods

### 8.2.1. Source and infection status of animals

The source of experimental sheep, the husbandry conditions under which they were maintained and the dose of *M. paratuberculosis* used to infect the sheep orally have been described in Chapters 4 and 5. Briefly, 10 sheep, aged 2 years, infected orally with approximately $3.4 \times 10^9$ *M. paratuberculosis* organisms within their first month of life (Group A) and 9 sheep, aged 1 year, infected orally with approximately $4.4 \times 10^8$ *M. paratuberculosis* organisms when 1.0-1.5 months of age (Group B), were used in this study. In addition, 10 uninfected sheep, aged 13-14 months, obtained as lambs from a flock where paratuberculosis had not previously been diagnosed, constituted the negative control group (Group C). All sheep were killed by intravenous injection of pentobarbitone. Animals in Group A were sacrificed 108 weeks after infection, and animals in Group B were killed 53 weeks postinfection.

The infection status of the 19 sheep infected experimentally with *M. paratuberculosis* and 10 uninfected control sheep was determined by histology and a PCR assay on samples of ileum and ileocaecal lymph node at the time of necropsy, as described in Chapters 4 and 5.

### 8.2.2. Collection of samples for immunological tests

***Blood samples***

Two to seven days prior to euthanasia, blood was collected from the jugular vein of each sheep into one 4 ml plain evacuated tube for serological tests and into two 4 ml

vacutainer tubes containing lithium heparin for the interferon-γ (IFN-γ) assay.

***Samples of prescapular lymph node for IFN-γ assay***

Immediately after euthanasia and prior to necropsy, 2 true-cut biopsy specimens (2 mm thick and 5 mm in diameter) of the prescapular lymph node (PLN) were collected from each sheep using sterile instruments and aseptic technique. After collection, each of the specimens was transferred to an Eppendorf tube containing 1 ml of RPMI-1640 (GIBCO BRL, USA) enriched medium (Gulle *et al.,* 1995) supplemented with penicillin (100 U/ml), and kept on ice before further processing.

## 8.2.3. Processing of samples for immunological tests

***Serum for serological testing***

Processing of blood samples for serology was as described in Chapters 4 and 5.

***Plasma for IFN-γ assay***

Processing of blood samples for the IFN-γ assay was performed as described in Chapter 5, except that 1.5-ml aliquots of each heparinised blood sample were dispensed into each of 4 polypropylene tubes. Two tubes were stimulated with antigen by adding 15 µl PBS containing 15 µg Johnin purified protein derivate (Johnin PPD) from *M. paratuberculosis* 316F (Central Veterinary Laboratory, Weybridge, England) to each tube, and 15 µl of PBS was added to each of the remaining 2 tubes (nil antigen control). The tubes were capped, gently mixed, and incubated in humidified atmosphere of 5% $CO_2$ at 37°C. One batch of Johnin PPD-stimulated and nil antigen control tubes was incubated overnight (17-19 h) and the second batch for approximately 48 h (46-50 h). After incubation, the tubes were centrifuged for 5-10 min at 1,800-2,400 g, and then plasma was harvested and stored at -70°C for subsequent testing. Each heparinised blood sample was processed within 6-7 h of collection.

***Processing of samples of prescapular lymph node for IFN-γ assay***

Within 6-8 h of collection each of the duplicate of PLN biopsy specimens was dispensed into an appropriate well on the 24-well tissue-culture plate (Nunc, Denmark)

containing 1.5 ml of RPMI-1640 enriched medium supplemented with penicillin. To one well, 30 µl PBS containing 30 µg Johnin PPD (Central Veterinary Laboratory, Weybridge, England) was added, and 30 µl of PBS was added to the other (nil antigen control). In order to facilitate perfusion of the antigen into the tissue, the plates were gently shaken for 2 min at 170 rpm on a minishaker, and then incubated in humidified atmosphere of 5% $CO_2$ at 37°C. Aliquots (200 µl) of the supernatant were collected from each well after 24 h (20-24) and 48 h (44-50) of incubation. After the first collection, the volume of medium in each well was reconstituted by adding 200 µl of medium alone to each of the control wells and 200 µl of the medium containing 4 µg Johnin PPD to each of the test wells. The samples of harvested supernatant were stored at -70°C for subsequent testing.

The enumeration and assessment of viability of cells in PLN samples was not performed. The reason for this was three-fold. Firstly, it was decided to preserve the mileu of the lymph node that would be disrupted by the standardisation procedures. Secondly, some of the cells, especially dendritic cells which are susceptible to shear forces (Hunt, 1987), could be damaged during the process of isolation. Thirdly, if the conventional enumeration and assessment of viability of cells was attempted the time lapse between collection of samples and stimulation with Johnin PPD would have been elongated and could be counterproductive. Instead, to ensure high viability and an adequate number of cells, samples of large volume were used and processed expeditiously within 6-8 h after collection.

### 8.2.4 Immunological testing

***Serological tests***

The complement fixation test (CFT), agar gel diffusion test (AGID) and enzyme-linked immunosorbent assays (ELISA) were performed, and results validated, as described in Chapters 4 and 5. Since the ELISA assay was developed for the detection of bovine antibodies, the cut-off value of 0.1 plus the mean of negative controls, as recommended by the manufacturer, and a low cut-off value of 0.05 were used to interpret the results. Duplicate samples of serum were tested. A sample was considered positive

when its mean optical density (OD) value, read at 450 nm, was equal to or greater than the predetermined cut-off.

***IFN-γ assay***

The IFN-γ assay (Bovigam ™, CSL, Australia) was performed, on single samples of plasma and prescapular lymph node, at the Central Animal Health Laboratory, Upper Hutt, New Zealand. The results of each plate were considered valid if the mean of the negative controls was <0.13, the mean of the positive controls was >0.7 and the positive control values had less than 30% deviation from their mean absorbance.

The cut-off value of 0.05, as recommended by the manufacturer, was used to interpret the results of the IFN-γ assay. The OD results, read at 450 nm, of nil antigen and Johnin PPD-stimulated samples of each animal were compared. A result was considered positive when the difference between the OD of Johnin PPD-stimulated sample and OD of nil antigen sample was equal to or greater than the predetermined cut-off.

In order to facilitate statistical analysis, the OD results were converted to indices using the following formula:

$$\gamma\text{-IFN index} = \frac{\text{OD Johnin PPD stimulated sample} - \text{OD nil antigen sample}}{\text{mean OD plate positive controls} - \text{mean OD plate negative controls}}$$ (Bassey and Collins, 1997)

In addition, for comparative purposes, OD values of Johnin PPD-stimulated and nil antigen samples were adjusted by dividing them by the mean OD of the plate's positive controls.

## 8.2.5. Statistical analysis

Data presented in Table 8.1 were analysed using the Fisher exact test. Comparison of IFN-γ indices and adjusted OD values between incubation times and between blood-based and PLN-based IFN-γ assays was performed by the Wilcoxon signed rank test and the Kruskal-Wallis/Dunn's multiple comparison test.

## 8.3. Results

### 8.3.1. Infection status of animals

Of 19 sheep that had been inoculated orally with *M. paratuberculosis*, 2 had clinical paratuberculosis confirmed by histology and the PCR on samples of ileum and ileocaecal lymph node. The remaining 17 sheep were clinically normal, however, 3 of these 17 sheep were infected with *M. paratuberculosis* as determined by histology and/or the PCR assay. The remaining 14 clinically normal sheep and 10 controls were tested negative by PCR and had no histological evidence of infection in sections of intestine or mesenteric lymph node.

### 8.3.2. Comparison of numbers of sheep detected by immunological tests

The numbers of sheep detected by the CFT, ELISA, AGID and the IFN-γ assay on samples of blood and prescapular lymph node (PLN) in the 19 sheep infected experimentally with *M. paratuberculosis* and in the group of 10 uninfected control sheep are presented in Table 8.1.

At the 0.05 cut-off value, the PLN-based IFN-γ assay on samples incubated overnight was positive in 16 of 19 experimentally infected sheep (84.2%), while 18 animals (94.7%) were detected by the PLN-based IFN-γ assay on samples incubated for 48 h. The ELISA at 0.1 cut-off gave positive reactions in 3 sheep (15.8%), the CFT in 4 sheep (21%) and the ELISA at 0.05 cut-off in 6 sheep (31.6%). Seven sheep (36.8%) tested positive by the blood-based IFN-γ assay on samples incubated overnight. The AGID and the blood-based IFN-γ assay on samples incubated for 48 h detected 8 sheep (42.1%). The total numbers of sheep tested positive by each of the PLN-based IFN-γ assays were significantly ($P \leq 0.017$) higher than those detected by any of the serological tests or the blood-based IFN-γ assays (see Appendix D).

None of the control sheep tested positive by the CFT, AGID, and ELISA at 0.1 cut-off.

Similarly, no positive reaction was obtained in these sheep by the PLN-based IFN-γ assay on samples incubated overnight or by any of the blood-based IFN-γ assays. One control sheep gave a positive reaction in the ELISA at 0.05 cut-off and one sheep was identified as positive by the PLN-based IFN-γ assay on samples incubated for 48 h.

**Table 8.1. Numbers of sheep tested positive by the AGID, CFT, ELISA and the blood-based and prescapular lymph node (PLN)-based IFN-γ assay. Nineteen sheep were experimentally infected with *M. paratuberculosis*. Ten uninfected sheep were used as negative controls.**

| Group of sheep | Number of sheep tested positive by immunological tests | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Serological tests | | | | Blood-based IFN-γ | | PLN-based IFN-γ | |
| | | | | | Sample incubation time | | Sample incubation time | |
| | CFT | AGID | ELISA 0.1 cut-off | ELISA 0.05 cut-off | ON | 48 hours | ON | 48 hours |
| A (n=10) | 0 | 3 | 1 | 1 | 2 | 1 | 8 | 9 |
| B (n=9) | 4 | 5 | 2 | 5 | 5 | 6 | 8 | 9 |
| Total A+B (n=19) | 4 | 8 | 3 | 6 | 7 | 7 | 16 | 18 |
| C (n=10) | 0 | 0 | 0 | 1 | 0 | 0^ | 0^ | 1 |

**A** 10 sheep inoculated orally with *M. paratuberculosis* 108 weeks before sampling

**B** 9 sheep inoculated orally with *M. paratuberculosis* 53 weeks before sampling

**C** 10 uninfected control sheep

**ON** overnight

**^** nine sheep tested

**PLN** prescapular lymph node

The effect of lowering of the cut-off values of the IFN-γ assays is presented in Table 8.2. The lowering the blood-based IFN-γ assay's cut-off value from 0.05 to 0.02 was associated with only a slight increase in the number of sheep detected by the tests. At the same time there was a gradual increase in the number of control sheep tested positive by the IFN both blood- and PLN-based assays.

**Table 8.2. The effect of lowering of cut-off values on numbers of sheep tested positive by the blood-based and prescapular lymph node (PLN)-based IFN-γ assay. Nineteen sheep were experimentally infected with *M. paratuberculosis*. Ten uninfected sheep were used as negative controls.**

| Group of sheep | Sample/ incubation time | Number of sheep tested positive by IFN-γ assay at various cut-off levels | | | |
|---|---|---|---|---|---|
| | | 0.05 cut-off | 0.04 cut-off | 0.03 cut-off | 0.02 cut-off |
| A (n=10) | Blood/ON | 2 | 3 | 3 | 4 |
| | Blood/48 hours | 2 | 2 | 2 | 3 |
| | PLN/ON | 8 | 8 | 8 | 9 |
| | PLN/48 hours | 9 | 9 | 9 | 9 |
| B (n=9) | Blood/ON | 5 | 5 | 5 | 5 |
| | Blood/48 hours | 6 | 6 | 6 | 8 |
| | PLN/ON | 8 | 8 | 9 | 9 |
| | PLN/48 hours | 9 | 9 | 9 | 9 |
| Total (A+B) (n=19) | Blood/ON | 7 | 8 | 8 | 9 |
| | Blood/48 hours | 8 | 8 | 8 | 11 |
| | PLN/ON | 16 | 16 | 17 | 18 |
| | PLN/48 hours | 18 | 18 | 18 | 18 |
| C (n=10) | Blood/ON | 0 | 2 | 4 | 5 |
| | ^Blood/48 hours | 0 | 0 | 0 | 2 |
| | ^PLN/ON | 0 | 0 | 2 | 3 |
| | PLN/48 hours | 1 | 1 | 2 | 2 |

**A** 10 sheep inoculated orally with *M. paratuberculosis* 108 weeks before sampling
**B** 9 sheep inoculated orally with *M. paratuberculosis* 53 weeks before sampling
**C** 10 uninfected control sheep
**ON** overnight
**^** nine sheep tested

### 8.3.3. Comparison of production of IFN-γ in samples of blood and prescapular lymph nodes at various times of incubation

As presented in Figure 8.1, in 19 sheep infected experimentally with *M. paratuberculosis* the median IFN-γ responses (IFN-γ indices) were significantly ($P<0.05$) higher in PLN samples than in blood samples incubated for similar periods of time. The analysis of the production of IFN-γ in individual groups of sheep revealed that the significant differences were mainly due to the high IFN-γ response in PLN samples from the 10 sheep that were experimentally infected 108 weeks before sampling.

**Figure 8.1.** IFN-γ indices in samples of blood and prescapular lymph node from 19 sheep infected experimentally with *M. paratuberculosis*. Of the 19 sheep, 10 were infected 108 weeks before sampling (Group A) and 9 were infected 53 weeks before sampling (Group B). Ten uninfected sheep were used as controls (Group C). Samples of blood and prescapular lymph nodes were incubated with 10 μg/ml and 20 μg/ml of Johnin PPD, respectively. Bars indicate medians.

**ON**-overnight.

In 19 experimentally infected sheep the median production of IFN-γ was significantly (P=0.0014) higher in PLN samples incubated for 48 h than in PLN samples incubated overnight. In comparison, the incubation of blood samples of these animals for 48 h had minimal effect on IFN-γ response.

**Figure 8.1.**

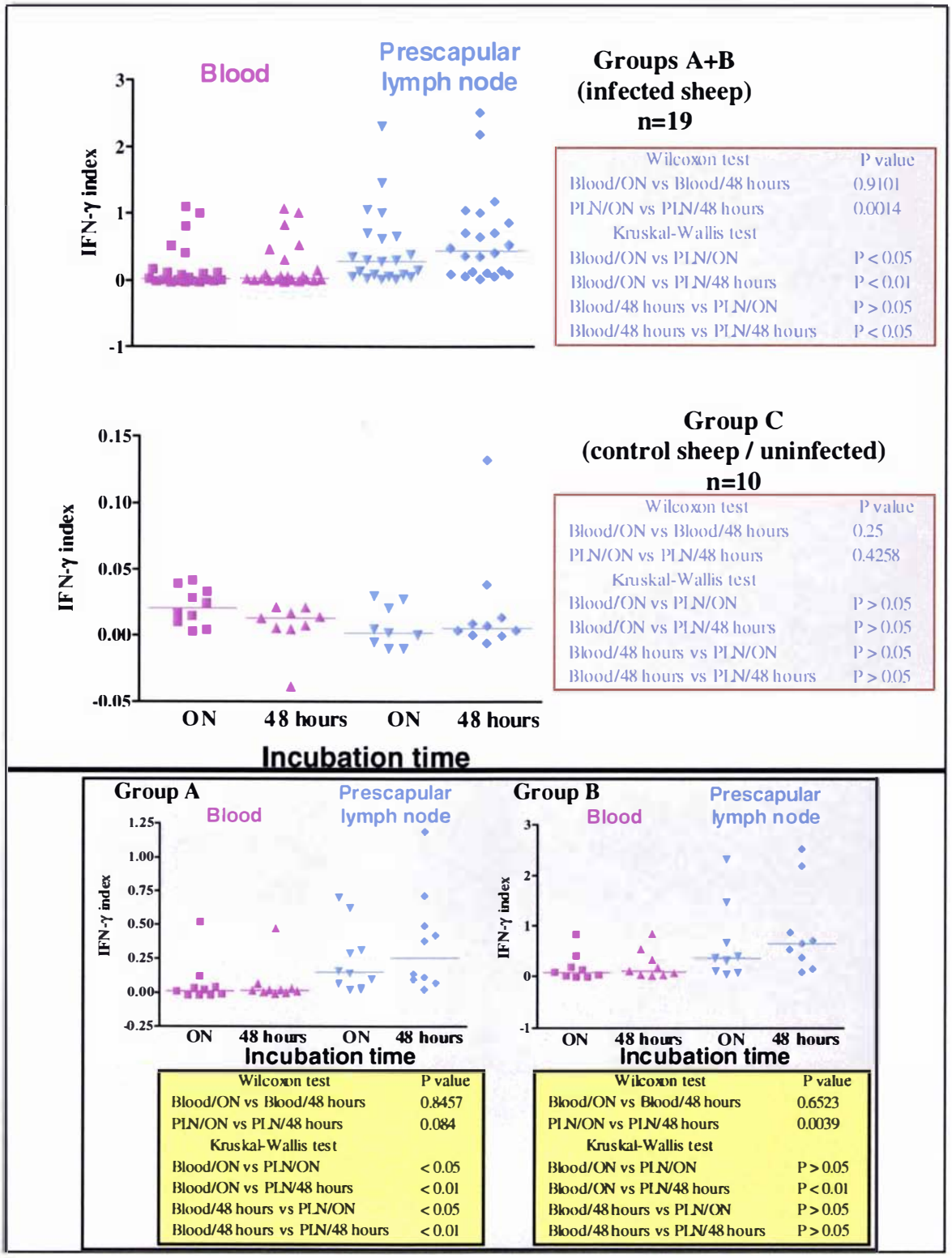
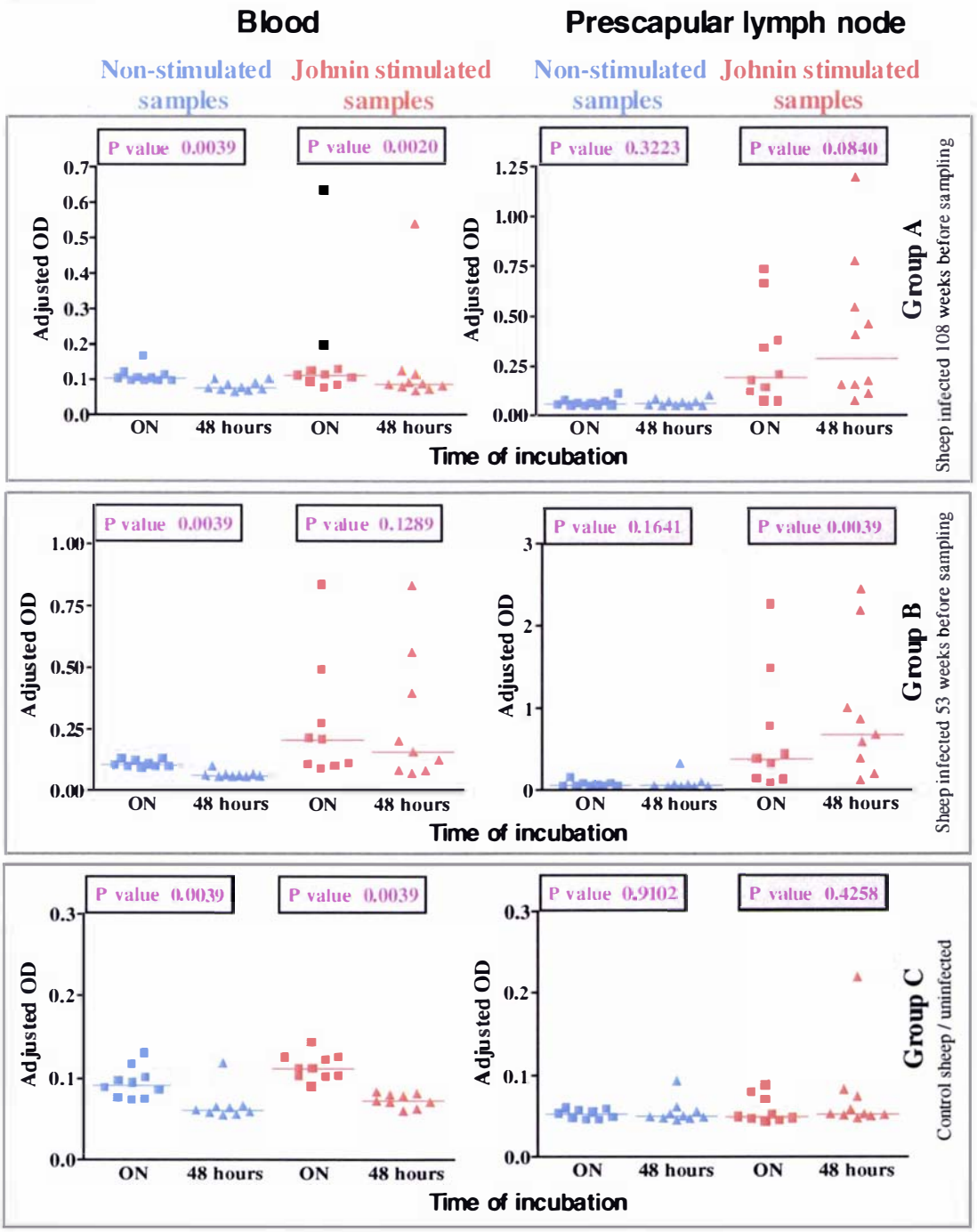


**Figure 8.2.** Adjusted OD values of the IFN-γ assay in samples of blood and prescapular lymph node from 19 sheep infected experimentally with *M. paratuberculosis*. Of the 19 sheep, 10 were infected 108 weeks before sampling (Group A) and 9 were infected 53 weeks before sampling (Group B). Ten uninfected sheep were used as controls (Group C). Samples of blood were incubated with 15 µl of PBS (non-stimulated) or with 10 µg/ml of Johnin PPD (Johnin stimulated). Samples of prescapular lymph node were incubated with 30 µl of PBS (non-stimulated) or with 20 µg/ml of Johnin PPD (Johnin stimulated). Data were analysed using the Wilcoxon signed rank test. Bars indicate medians.

**ON**-overnight.

As presented in Figure 8.2, in all groups of sheep, there was a general trend for the OD values to be lower in both non-stimulated and antigen-stimulated blood samples when they were incubated for 48 h. In contrast, OD values in antigen-stimulated PLN samples of experimentally infected sheep were higher when the samples were incubated for 48 h. The extension of the incubation time to 48 h had little effect on OD values in non-stimulated PLN samples from these sheep and on OD values in both antigen-stimulated and non-stimulated PLN samples from the control sheep.

**Figure 8.2.**



## 8.4. Discussion

In the present study, 84.2% of sheep infected experimentally with *M. paratuberculosis* were identified as positive by the IFN-γ assays on samples of the prescapular lymph node (PLN) incubated overnight, and 94.7% were detected when the PLN-based IFN-γ assay was applied to samples incubated for 48 hours. Significantly ($P \leq 0.017$) more sheep were detected by the PLN-based IFN-γ assays than by any of the serological tests or the blood-based IFN-γ assays. This is an interesting finding because only 2 of the 19 experimentally infected sheep had clinical paratuberculosis and the remaining 17 sheep were clinically normal. Furthermore, 14 of the 19 sheep had no histological evidence of paratuberculosis and samples of ileum and ileocaecal lymph node from these animals tested negative by PCR at the time of collection of the PLN samples. As described in the previous chapter, 12 of these 14 histologically-negative sheep showed immunological evidence of infection on more than one occasion during the period between experimental infection with *M. paratuberculosis* and collection of the PLN samples at necropsy. In the light of this data, most of the sheep should be regarded as subclinically infected. Considering the time lapse between experimental infection and collection of PLN samples, it is highly likely that some sheep may have completely recovered from the infection. Therefore the positive reactions obtained by the PLN-based IFN-γ assay in these sheep may represent an immunological indication of the exposure to the organism rather than active infection.

The high detection rate of the PLN-based IFN-γ assay is a reflection of high IFN-γ response in antigen-stimulated samples. There are several possible explanations for the production of IFN-γ being significantly ($P<0.05$) higher in PLN samples than in blood samples incubated for similar periods of time. Firstly, as the concentration of Johnin PPD was 10 μg/ml of blood and 20 μg/ml of PLN sample, it could be due to higher amount of antigen used to stimulate cells in the PLN samples. Secondly, since preferential migration of antigen-specific memory T cells to peripheral lymph nodes has previously been reported in sheep (Premier *et al.*, 1996), it is likely that in the present study the high magnitude of IFN-γ response in the PLN samples represents Johnin PPD-induced stimulation of such cells. Thirdly, it may be due to more efficient antigen presentation in the PLN samples, as dendritic cells, that are believed to be the most efficient

antigen presenting cells (Inaba *et al.,* 1990; Steinmann, 1991), are predominantly located in the lymph nodes. Fourthly, since blood samples were incubated in tubes made of polypropylene, it is also possible that this material had an adverse effect on antigen presentation. In addition, although cells in the PLN samples were not enumerated, considering the volume of samples used, it could be expected that larger numbers of leukocytes were stimulated in the PLN samples than in the blood samples. It is also possible that, the high IFN-γ response in the PLN samples may represent an immunological reaction due to exposure to bacteria that share common epitopes with *M. paratuberculosis*. This seems unlikely in the light of results obtained in the group of control sheep. The experimentally infected sheep and control animals were kept on the same farm and both groups had probably been exposed to similar bacterial flora. However, as mentioned before, in order to preserve the mileu of the lymph node, limit damage of cells and shorten the time lapse between collection of samples and stimulation with Johnin PPD, cells in the PLN samples were neither enumerated nor standardised for viability. Therefore, there is a remote chance that the results obtained in the group of control sheep were due to either poor viability or inadequate number of cells in PLN samples. Since large volume samples were processed expeditiously, problems associated with reduced viability or inadequate number of cells seem unlikely.

An increase in the production of IFN-γ, when peripheral blood mononuclear cells from cattle with naturally acquired paratuberculosis were stimulated with *M. paratuberculosis* antigens for 48 hours, has previously been reported (Stabel, 1996). A similar trend was also observed in the present study in PLN samples from sheep infected experimentally with *M. paratuberculosis*. In contrast, the incubation of blood samples from these animals for 48 hours had minimal effect on the levels of IFN-γ. This could be due to lower amount of antigen used to stimulate blood samples, release of factors from blood cells that interfere with T-cell activation upon extended incubation, reduced viability of T-cells or faster degradation of IFN-γ in blood. Alternatively, it may represent insufficient number or type of cells to maintain steady production of IFN-γ for prolonged period of time, exhaustion of T cells, or inadequate antigen presentation upon extended incubation.

When the PLN-based IFN-γ assay was applied to samples incubated for 48 hours a posi-

tive reaction was obtained in one of 10 control sheep. This may represent either a non-specific reaction or exposure to *M. paratuberculosis*, as occasionally, the control sheep shared grazing with sheep of unknown infection status.

In summary, the present study has demonstrated that the production of IFN-γ above the commonly used cut-off level can be detected in samples of prescapular lymph node from sheep infected experimentally with *M. paratuberculosis*. Since a majority of these sheep, in the light of data derived from histology and the PCR, could be considered subclinically infected, this indicates the potential utility of the PLN-based IFN-γ assay using fine needle aspirate in live animals for the detection of sheep exposed to *M. paratuberculosis*. However, with such a small number of animals tested, it is difficult to comment definitively on the diagnostic performance of the test and more data are required from live animals. In addition, there is a need for further standardisation and optimisation of the incubation procedure in respect to the number of cells and amount and type of antigen(s) used for stimulation of samples.

# Chapter 9

# General discussion

Paratuberculosis is an infectious, chronic, wasting disease of domestic and wild ruminants found throughout the world. A recent review of New Zealand Ministry of Agriculture and Food records revealed that by May 1993 the disease had been confirmed in 5.2% of sheep flocks and 6.2% of cattle herds. These figures are assumed to grossly underestimate the prevalence of the disease in New Zealand (Brett, 1998). The aetiologic agent of paratuberculosis shares considerable DNA homology with *Mycobacterium avium,* and it has been proposed by Thorel *et al.* (1990) that the agent should be classified as *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*).

## 9.1. Development and evaluation of a PCR assay for the detection of *Mycobacterium paratuberculosis* in tissue samples

Programmes aimed at controlling paratuberculosis are based on either vaccination, or detection and culling of infected animals. Attempts to control the disease by the latter method have been hampered by a lack of suitable diagnostic tests. Although the isolation of *M. paratuberculosis* from an animal provides the definitive diagnosis of infection with the organism, the prolonged isolation time and difficulty in growing some strains (Gunnarsson, 1979; Brooks *et al.,* 1988; Shulaw *et al.,* 1993; Collins *et al.,* 1993a) limit the use of this technique. The advent of the polymerase chain reaction (PCR) and the identification of IS*900*, an atypical sequence considered unique for *M. paratuberculosis* (Green *et al.,* 1989) have provided an opportunity for rapid and accurate identification of this organism.

When this study was embarked on, attempts to use the PCR for detecting *M. paratuberculosis* in faeces had not been encouraging (Sockett *et al.,* 1992; van der

Giessen *et al.,* 1992; Collins *et al.,* 1993a; Collins *et al.,* 1993b), presumably due to the presence of inhibitory substances in faecal samples (Vary *et al.,* 1990; van der Giessen *et al.,* 1992) and the small volume of samples (van der Giessen *et al.,* 1992). Although numerous authors have reported the isolation of *M. paratuberculosis* from various extra-intestinal organs and body fluids of infected animals (Goudswaard, 1971; Hines *et al.,* 1987; Seitz *et al.,* 1989; Rohde and Shulaw, 1990; Sweeney *et al.,* 1992a; Koenig *et al.,* 1993; Streeter *et al.,* 1995), the diagnostic potential of culturing the organism from specimens other than ileocaecal lymph node has been considered to be insignificant (Koenig *et al.,* 1993). Recently, the application of the PCR to specimens other than sputum and faeces for diagnosing mycobacterial infections has been given credibility through the detection of *M. tuberculosis, M. avium,* and *M. intracellulare* by this method in the blood of human patients (Iralu *et al.,* 1993; Schluger *et al.,* 1994; Rolfs *et al.,* 1995) and in lung tissue from a sheep with paratuberculosis (Bassett *et al.,* 1993).

In order to assess the potential utility of the PCR for detecting *M. paratuberculosis* in tissue samples, a PCR system, based on the primers 90 and 91 described by Sanderson *et al.* (1992), was developed. The identity of the 400-bp PCR products generated by primers 90 and 91 was confirmed by hybridisation with a 194-bp probe, a product of internal primers JG1 and JG2. With purified extracts of DNA, the PCR assay was found to be both highly sensitive and specific. This assay was capable of detecting approximately 1 fg of *M. paratuberculosis* DNA, a detection limit that is comparable with other studies in which DNA from *M. bovis* was extracted in a similar way to that used in the current study (Wards *et al.,* 1995). Among 30 bacterial species tested in this study, only DNA from *M. scrofulaceum* showed cross-reactivity. Others have also found cross-reactions with *M. scrofulaceum* in IS*900*-based PCR and suggested that IS*900*-related sequences may occur in this organism (Thoresen and Olsaker, 1994). Alternatively, the DNA sample of *M. scrofulaceum* used in the current study might have been contaminated with *M. paratuberculosis* DNA. Due to limited time and resources, the origin of the cross-reactivity with this organism was not investigated further. *M. scrofulaceum* is not considered to be a primary pathogen, although it has occasionally been isolated from human patients with AIDS (Campos-Herrero *et al.,* 1996; Horsburgh, 1996), children with lymphadenitis (Pang, 1992; Turneer *et al.,* 1994) and immunocompromised mice (Ueda *et al.,* 1992). To our knowledge, this organism has never been isolated from sheep, and the possibility of *M. scrofulaceum* causing false positive results in clinical

samples from sheep seems remote.

Following the optimisation of the PCR assay, 2 studies were designed to evaluate its use on clinical specimens. In the first study, the assay was applied to samples of DNA from solid tissues and blood of 20 sheep suspected of having paratuberculosis. The detection rate of *M. paratuberculosis* DNA, when results of single tests were interpreted in duplicate, was 72% for ileocaecal lymph node, 90% for liver, and 100% for ileum in sheep with histologically confirmed paratuberculosis. A single PCR test detected the target DNA in 66% of blood samples. As the assay was shown to be highly specific, these results raised some hope for the use of a PCR-based test in the diagnosis of paratuberculosis. However, due to a limited number of animals tested it was not possible to draw firm conclusions on the diagnostic performance of the test.

To further assess the PCR, the assay was applied to liver biopsy specimens and samples of blood collected sequentially from 14 sheep infected experimentally with *M. paratuberculosis* at the age of 1-1.5 months. Results of this study indicate that the PCR assay, when performed on samples of blood and liver, does not enhance detection of *M. paratuberculosis* in subclinically infected sheep, or in clinically affected sheep with mild or moderate extra-intestinal infection. As in the first study, the PCR assay was found to be highly specific, but among 117 blood samples collected sequentially, over 53 weeks, from experimentally infected sheep, only 2 tested positive by PCR. Although these positive results were obtained in 2 sheep with histologically confirmed paratuberculosis, the assay failed to detect the target DNA in blood samples from the remaining 5 sheep that subsequently had paratuberculosis confirmed by histology. Likewise, only 2 of 7 sheep with histological evidence of paratuberculosis were detected by the PCR assay on liver biopsy specimens collected 32 weeks after oral infection with *M. paratuberculosis*.

The failure to consistently detect *M. paratuberculosis* DNA in liver biopsy specimens and blood samples may reflect absence of the target DNA in these tissues during the collection times. Alternatively, it might have been due to either inadequate sensitivity of the PCR assay, insufficient volume of samples or the presence of PCR inhibitory substances in the samples. Unexpectedly, no positive PCR reaction was given by blood

samples that were collected from the experimentally infected sheep around the time when these animals were showing clinical signs consistent with paratuberculosis. This is in contrast with the high detection rate of the target DNA in blood samples from naturally infected sheep with clinical paratuberculosis. The discrepancy is likely to be due to less severe extraintestinal infection in the experimentally infected animals than this observed in the naturally infected sheep.

The results presented in this thesis seem to further illustrate the limitations of this new technology in the diagnosis of paratuberculosis in live animals. Furthermore, sensitivity of the PCR assay when it was applied to samples of ileum and ileocaecal lymph node both from sheep suspected of having naturally acquired paratuberculosis and experimentally infected animals was similar to that achieved by histology. This suggests that the PCR assay offers little advantage over histology in the routine post-mortem diagnosis of paratuberculosis. However, the rapid availability of PCR results and opportunity to identify strains that are difficult to culture are the major advantages of this technique. The decrease in sensitivity of the PCR when it was applied to ovine liver DNA spiked with *M. paratuberculosis* DNA exemplifies the well recognised inhibition of the amplification process by extraneous substances present in DNA samples (Lo *et al.,* 1989; Rossen *et al.,* 1992). As mentioned above, the lack of sensitivity of the PCR may also be due to the small volume of sample. Thus, future attempts to increase sensitivity of the PCR should be focused on improved methods of recovery and purification of target DNA from large-volume clinical samples. The recently reported hybridisation-capture PCR method (Millar *et al.,* 1995) offers some prospects for such improvement. An alternative approach consists of isolating *M. paratuberculosis* from clinical samples in the BACTEC radiometric culture system, followed by PCR-based identification of the mycobacterial growth. The BACTEC-PCR combination has been shown to be both more sensitive and rapid than routine culture (Cousins *et al.,* 1995; Whittington *et al.,* 1998). The immunomagnetic separation of *M. paratuberculosis* organisms from large-volume clinical samples in conjunction with an IS*900*-based PCR may also prove to be a useful diagnostic technique (Grant *et al.,* 1998).

Theoretically, the PCR technique is able to detect a single copy of target DNA. Although in practice this detection level is rarely achieved, the amplification power of the PCR is both its strength and weakness, as it makes the technique prone to problems

associated with contamination. The occasional suspicious results (presence of a signal on a dot blot but absence of the band of 400 base pair PCR products on a gel) obtained in 2 samples from 2 control animals, one in a sample of blood DNA and one in a liver biopsy specimen, are difficult to explain but may be due to either non-specific cross-reactivity detectable by hybridisation or contamination. Alternatively, since the control animals were exposed to sheep of unknown infection status, this suspicious reaction may represent a low level of natural infection. Due to limited resources, the use of the uridine triphoshate / uracil-N-glycolase (UTP/UNG) system, as a measure to minimise contamination problems, was not attempted in this study. Nevertheless, the results presented in Chapters 3 and 6 suggest that the adherence to strict physical separation of pre- and post-amplification steps was sufficient to control contamination problems.

## 9.2. Comparison of immunological tests for the diagnosis of paratuberculosis in sheep

As previously mentioned, the isolation of *M. paratuberculosis* from an animal provides the definitive diagnosis of infection with the organism. Although serological tests for paratuberculosis provide only indirect evidence of infection, their rapid availability is the major advantage they have over culture.

Diagnostic performance of a test has two components, sensitivity and specificity. Sensitivity of a serological test depends on the concentration of antibodies required to give a positive result, and is therefore affected by the kinetics of the humoral response. The two experimental trials presented here created an opportunity to study the effect of antibody kinetics on the diagnostic performance of three commercially available serological tests for paratuberculosis: complement fixation test (CFT), agar gel immunodiffusion test (AGID) and enzyme-linked immunosorbent assay (ELISA).

In the past, the AGID test was generally recommended as the most efficient serological test for paratuberculosis in sheep (Julian, 1975; de Lisle and Wall, 1983). Furthermore, this antibody assay has recently been reported to be the most specific test, with reasonable sensitivity, for identifying sheep in the later stages of the disease (Dubash *et al.*,

1996). The consistent and relatively high detection rates of the AGID during both trials presented here, along with relatively short median detection times by which this test identified as positive 50% of experimentally infected sheep, support these recommendations. In both trials, detection rates of the ELISA assay, at the recommended 0.1 cut-off value, were lower than that of the AGID and CFT. The ELISA is intended for use in cattle and better accuracy might be expected with a test developed specifically for sheep. Alternatively, a lower cut-off value could be used. In the present study, there was an increase in the detection rate of the ELISA when the results were interpreted at a 0.05 cut-off. However, since seroconversion occurs late in the course of infection, such an assay may not have a great impact on the diagnosis of ovine paratuberculosis. The prolonged median detection times of the CFT indicate that this test detects sheep in the more advanced stage of infection. This is in agreement with previous data (Hilbink *et al.,* 1994), and suggests that the CFT is less likely to be suitable for detecting subclinically infected sheep.

Although the results presented in this thesis suggest that the antibody assays are highly specific, none of the serological tests was able to detect all sheep, both experimentally infected and with naturally occurring paratuberculosis, which had histologically confirmed paratuberculosis. The inability of antibody assays to identify animals which pose the greatest risk of spreading the infection supports the general opinion that serological tests have limited value for the control of ovine paratuberculosis (Hilbink *et al.,* 1994).

In contrast, the whole-blood IFN-γ assay, which was assessed only during the second trial, detected all 14 experimentally infected sheep by the 27th week after infection. This assay detected significantly more sheep, and over shorter period of time, than any of the serological tests. Furthermore, its specificity was high and similar to that achieved by the antibody assays. As such, further investigation of the use of the IFN-γ assay for detection of sheep infected with *M. paratuberculosis* is warranted.

In the blood of some sheep infected experimentally with *M. paratuberculosis*, the antigen-specific IFN-γ response could be detected above the predetermined cut-off level only during a relatively short period after exposure to the organism. Premier *et al.* (1996) have reported, that in sheep antigen-specific memory T cells migrate preferen-

tially to peripheral lymph nodes, regardless of the route of immunisation. In order to investigate the potential utility of the IFN-γ assay on specimens of peripheral lymph node, the assay was applied to samples of prescapular lymph node (PLN) from 19 sheep infected experimentally with *M. paratuberculosis*.

The PLN-based IFN-γ assay on samples incubated overnight gave positive reactions in 84.2% of experimentally infected sheep, and 94.7% were detected when the IFN-γ assay was applied to PLN samples incubated for 48 hours. Significantly more experimentally infected sheep were detected by the PLN-based IFN-γ assays than by any of the serological tests or the blood-based IFN-γ assays. This is an interesting finding, because the majority of the sheep, in the light of the immunological data and results derived from histological examination and the PCR on samples of ileum and ileocaecal lymph node, could be regarded as subclinically infected. Furthermore, considering the time lapse between experimental infection and collection of PLN samples, it is highly likely that some sheep may have completely recovered from the infection. Thus, the positive reactions obtained by the PLN-based IFN-γ assay in these sheep may represent an immunological indication of the exposure to the organism rather than active infection.

Although the number of control animals used in the current study was too small to be conclusive, the data presented in Chapter 8 suggest that the PLN-based IFN-γ assay is also specific. This indicates the potential utility of the PLN-based IFN-γ assay, using fine needle aspirates in live animals, for the detection of sheep and cattle exposed to *M. paratuberculosis*. However, for both the blood-based and PLN-based IFN-γ assays, there is a need for further standardisation and optimisation of the incubation procedure in respect to the number of cells, and the amount and type of antigen(s) used for stimulation of samples. In addition, more data are required from live animals before making valid conclusions regarding the diagnostic performance of the test.

In the past, tests that measure the peripheral cell-mediated immune response in paratuberculosis, an intradermal and intravenous test, were reported to lack accuracy (Larsen *et al.,* 1963; Larsen and Kopecky, 1965; Merkal *et al.,* 1968b; de Lisle *et al.,* 1980a). It is believed that, despite the low incidence of clinical cases, in herds endemically infected with *M. paratuberculosis* most animals are exposed to the organism (Whitlock,

1992; Chiodini *et al.,* 1984b). As presented here and elsewhere (Brotherston *et al.,* 1961a; Kluge *et al.,* 1968), even some animals that had been inoculated experimentally with *M. paratuberculosis* did not develop the disease. Although a number of factors is likely to contribute to immunity in paratuberculosis, a positive association between the magnitude of peripheral cell-mediated response measured by skin test and ability to control the infection has previously been reported in sheep and cattle (Gilmour and Brotherston, 1966; Gilmour *et al.,* 1977; Wentnik *et al.*, 1993). This may provide an explanation for the apparent lack of accuracy of the skin test. In the present study, the magnitude of IFN-γ production was higher in blood of sheep infected experimentally with *M. paratuberculosis* in which acid-fast organisms (AFO's) were not detected than in experimentally infected animals in which AFO's were detected. This raises the question whether the IFN-γ assay is suitable for programmes to control paratuberculosis. Data presented here suggest that both the blood- and PLN-based IFN-γ assay should be useful for identification of animals exposed to *M. paratuberculosis.* Attempts to use this assay in control programmes that are based on testing and culling of positive reactors could result in the removal of animals that have successfully mounted an immune response to the infection. If this feature is under genetic influence, culling of this type could lead to a lowered flock resistance to disease.

Similarly to the IFN-γ assay, a positive reaction in any of the antibody assays used in this study could only be considered as an indication of exposure to *M. paratuberculosis.* Other workers have also reported poor discrimination between subclinically and clinically infected cattle by an ELISA assay and postulated the use of likelihood ratios to optimise the ELISA's discriminatory power (Spangler *et al.,* 1992b). However, large data sets and knowledge of the infection status of animals tested are required to calculate representative reference values for the likelihood ratios analysis and subsequent culling decisions. Despite these pitfalls and the possibility that in some cases the reference values may not be relevant for the populations tested, this method of interpretation of results has the advantage of being independent of the prevalence of infection, which is frequently unknown and difficult to estimate.

## 9.3. Vaccination against paratuberculosis of lambs already infected experimentally with *Mycobacterium paratuberculosis*

The induction of immunity against *M. paratuberculosis* through vaccination provides an alternative to test-and-cull programmes for controlling paratuberculosis. Since in endemically infected herds many animals are expected to be exposed to the infection in the neonatal period before vaccination (Chiodini *et al.*, 1984b), a longitudinal study was designed (Trial 2) to assess the usefulness of vaccination in sheep already exposed to *M. paratuberculosis*. This one year-duration study involved 28 lambs inoculated orally with *M. paratuberculosis*, 14 of which were vaccinated against paratuberculosis 2 weeks postinfection with a live-attenuated vaccine. Results of this study showed that infection was less severe, as determined by histology and the PCR on ileum samples, in the group of infected-vaccinated sheep. In addition, antigen-specific production of IFN-γ in blood and antibody levels in serum were detected earlier, and were greater throughout the study, in the infected-vaccinated group than in the infected-unvaccinated group. It is likely that the early onset and high magnitude of the immune response in infected-vaccinated sheep was responsible for the ability of a significant proportion of these animals to control the infection. Because the sheep were vaccinated 2 weeks after oral inoculation with *M. paratuberculosis*, it is likely that the infection was in an early stage when the vaccine was administered. Whether the vaccine would have any therapeutic value in animals with more advanced stages of infection remains to be determined. The use of *M. bovis* BCG and heat-killed *M. leprae* vaccine as a therapeutic agent in leprosy patients has been associated with reversal toward the tuberculoid form of leprosy in 90% of cases initially classified as lepromatous (Meyers *et al.*, 1988). Immunotherapy using various mycobacterial agents is currently considered to be a beneficial adjunct to chemotherapy for the treatment of multibacillary leprosy patients (Katoch, 1996).

According to Saxegaard and Fodstad (1985), the use of vaccine alone cannot control paratuberculosis. However, a significant reduction in mortality due to paratuberculosis has been reported in vaccinated sheep (Sigurdsson, 1960) and cattle (Schaik *et al.*, 1996). Others have shown that the infection rate, as determined by post-mortem examinations, was significantly lower in vaccinated goats (Saxegaard and Fodstad, 1985) and

cattle (Larsen *et al.,* 1978) than in unvaccinated animals. Thus, considering both the prevalence of the disease in New Zealand and limitations of currently available tests for the diagnosis of paratuberculosis, vaccination may be a more feasible control option than the test-and-cull approach. Data presented in this thesis are also in favour of the vaccination option, but the use of vaccine does have disadvantages. As reported here and elsewhere (Gilmour and Brotherston, 1966; Milestone, 1988), subcutaneous injection of vaccine is frequently associated with the development of large fibrocaseous lesions at the injection site. The extent of these lesions may limit the use of the vaccine. Furthermore, severe localised reactions after accidental self-inoculation present a human health risk. In order to minimise these side effects, alternative formulations or routes of vaccination should be investigated.

## 9.4. Experimental model of paratuberculosis in sheep

The high incidence of infection, and the similarity between lesions in sheep with naturally occurring paratuberculosis and those in sheep infected experimentally with *M. paratuberculosis* in Trial 1, indicated that the experimental model could be a suitable tool for the study of paratuberculosis. This model proved to be useful for the assessment of the protective or therapeutic value of a live-attenuated vaccine in sheep already infected with *M. paratuberculosis.* It may also be useful for further study of immune response at the site of infection and mechanisms of resistance to mycobacterial infection in the natural host.

## 9.5. Perineural lesions

An unexpected finding in the present study was the accumulation of mononuclear cells around intestinal nerves in both naturally and experimentally infected sheep with histological evidence of paratuberculosis. These neural lesions resembled changes observed in early stages of human leprosy and AFO's were detected within some of the mononuclear cells in 3 cases. The only neural lesions previously described in paratuberculous cattle are those which were characterised by infiltration of myenteric ganglions

with globule leukocytes (Buergelt *et al.,* 1978a). Such lesions were not observed in the current study, perhaps due to either species variation in the host response to infection or variation of strain pathogenicity.

Of importance is the question of whether the intestinal nerves or the nerve-associated cells, as occurs in *M. leprae* infections in people, were the primary target of the inflammatory response. In the present study, the neural lesions were not detected in all animals with histological evidence of paratuberculosis, nor were they generalised. Since nerves run parallel to lymphatics and granulomatous lymphangitis is a feature of paratuberculosis, the lesions could represent an extension of local lymphangitis and the nerve involvement is therefore secondary rather than primary. However, it is possible that nerves or the nerve-associated cells in the affected parts of intestine express stress-antigens (heat-shock proteins) that trigger an autoimune-like reaction. Furthermore, although AFO's were detected within the epi- and perineural mononuclear cells in only 3 sheep, the possibility of inflammatory response due to the presence of mycobaterial antigen(s) in the epineurium and perineurium in other cases cannot be excluded.

Regardless of the pathogenesis of the neural lesions, the possibility of interference with nerve functions and subsequent contribution to the pathogenesis of paratuberculosis should be considered. The importance of this phenomenon remains to be determined.

## 9.6. Conclusions

This study has demonstrated:

⇒ a protective value of a live attenuated vaccine in lambs infected experimentally with *M. paratuberculosis* 2 weeks prior to vaccination,

⇒ a potential use of a IFN-γ assay on blood or samples of prescapular lymph node for the detection of sheep infected with *M. paratuberculosis*,

⇒ the limitations of the PCR in the diagnosis of paratuberculosis,

⇒ the limited value of serological tests for the diagnosis and control of ovine paratuberculosis.

# Appendix A.1.

**Protocol for dot blot hybridisation and detection of PCR products (generated by 90 and 91 primers) using the DIG-labelled 194-bp probe**

For dot blot hybridisation, amplification mixtures in each PCR tube were heated for 4 min at 94°C, immediately transferred on ice, and then 1 µl aliquots of each reaction mixture deposited manually onto a Hybond-N membrane (Amersham, UK). After DNA cross-linking by ultraviolet light irradiation for 3.5 min, the membrane was hybridised for 2 h at 65°C in 7-10 ml of Rapid-hyb buffer (Amersham, UK) containing 5 ng of the DIG-labeled 194-bp probe per 1 ml of the buffer. After hybridisation, the membrane was washed under stringent conditions: once in 2xSSC, 0.1% SDS at room temperature for 10 min, followed by one wash in 1xSSC, 0.1% SDS at 65 °C for 10 min and finally in 0.7xSSC, 0.1% SDS at 65 °C for 15 min.

After the post-hybridisation washes, the membrane was equilibrated at room temperature for 1 min in 40 ml of washing buffer (10 mM maleic acid, 15 mM NaCl; pH 7.5, 0.3% (v/v) Tween®20) and blocked for 60 min at room temperature in 40 ml of blocking solution (10 mM maleic acid, 15 mM NaCl; pH 7.5, 1 % (w/v) blocking reagent). The blot was then incubated at room temperature for 30 min in freshly prepared antibody solution (40 ml of blocking solution to which 4 µl anti-digoxigenin antibody was added), washed twice (15 min per wash) in washing buffer, and equilibrated for 2 min in 40 ml of detection buffer (10mM Tris-HCL, 10 mM NaCl pH 9.5). After equilibration, the membrane was incubated at room temperature for 2 h in 40 ml of freshly prepared colour substrate solution containing detection buffer to which 4.5 µl of NBT solution (75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformide) and 3.5 µl of BCIP solution (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformide) was added per 1 ml of the buffer. The colour development was allowed to occur in the dark and was stopped by washing the membranes with 100 ml of TE for 10 min.

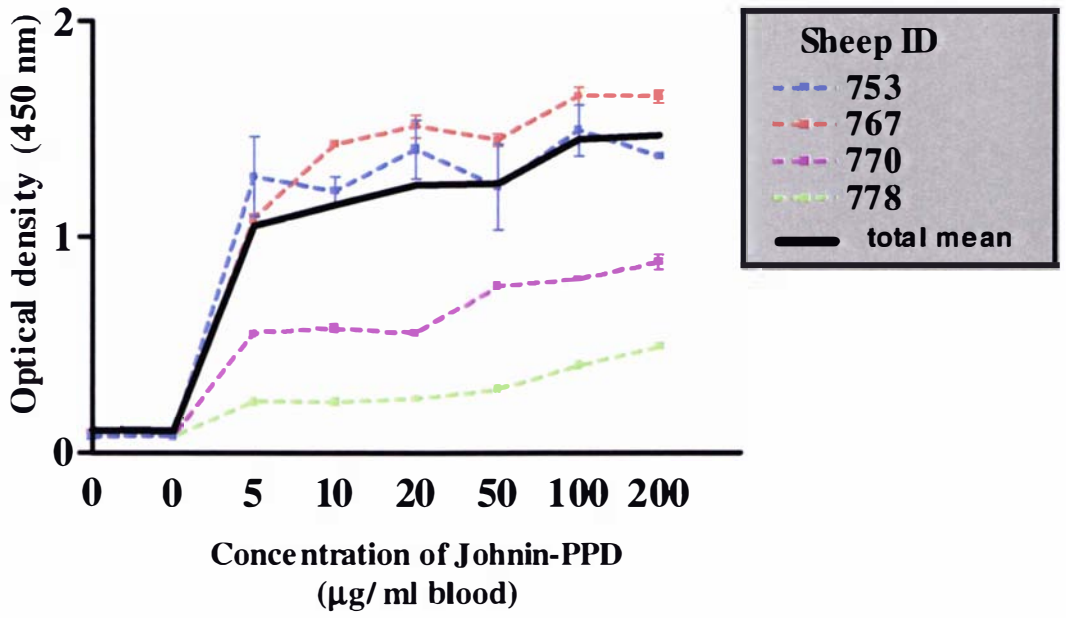
# Appendix A.2.

**Protocol of enzyme-linked immunosorbent assay (ELISA) for antibodies to *M. paratuberculosis* (Paracheck™)**

Aliquots (25 µl) of each serum sample, including 2-3 positive and 2-3 negative controls, were added to 475 µl of diluent buffer, mixed thoroughly and incubated at room temperature for 60 min. Duplicate 100 µl aliquots of the mixture were dispensed into appropriate wells. The plate was thoroughly shaken and incubated at room temperature for 30 min. After incubation, the diluted serum samples were discarded, the plate washed 6 times with wash buffer (diluted 1:20 with distilled water) and then aliquots (100 µl) of freshly prepared conjugate (Horseradish peroxidase labelled anti-bovine Ig diluted 1:100 with the conjugate buffer) were added to each well. The plate was shaken and incubated at room temperature for 30 min. The conjugate was then discarded and the plate washed 6 times with the wash buffer. After the final wash, the plate was drained and 100 µl aliquots of freshly prepared enzyme substrate (Chromogen diluted 1:100 with the enzyme substrate buffer) were dispensed into each well. The plate was shaken and the colour development was monitored by reading the absorbance of the positive control at 620 nm. The reaction was stopped by adding 50 µl of 0.5 M $H_2SO_4$ to each well when the absorbance of the positive control at 620 nm was between 0.35 and 0.40. Finally, the absorbance was read at 450 nm within 20 min of termination of the reaction.

# Appendix B.1.

**IFN-γ response to various concentration of Johnin-PPD in blood samples of 4 sheep infected orally with *M. paratuberculosis* as lambs.**




Heparinised blood samples (1.5 ml) from 4 sheep orally infected with *M. paratuberculosis* were stimulated with various amounts of Johnin-PPD (starting from 5 to 200 µg/ml of blood) and PBS. After overnight incubation at 37°C in humidified atmosphere of 5% $CO_2$, plasma was harvested and duplicate plasma samples from each animal were assayed for IFN-γ using a commercial bovine enzyme-linked immunosorbent assay (Bovigam™) according to the manufacturer's (CSL, Australia) recommendations. Data is presented as mean of duplicate samples ±SEM.

# Appendix B.2.

**Protocol of enzyme-linked immunosorbent assay for interferon-γ (Bovigam™)**

Duplicate aliquots (50 µl) of each test and control plasma samples were added to appropriate wells containing 50 µl of plasma diluent, mixed thoroughly and incubated at room temperature for 60 min. For each plate, 2 to 3 negative and 2 to 3 positive control samples were included. After the incubation, the diluted plasma samples were discarded, the plate washed 6 times with wash buffer (diluted 1:20 with distilled water) then drained. Aliquots (100 µl) of freshly prepared conjugate (Horseradish peroxidase-labelled anti-bovine IFN-γ antibody diluted 1:100 with the diluent buffer) were added to each well. The plate was shaken and incubated at room temperature for 60 min. The conjugate was then discarded, the plate washed 6 times with the wash buffer, drained, and 100 µl aliquots of freshly prepared enzyme substrate (Chromogen diluted 1:100 with the enzyme substrate buffer) were dispensed into each well. The plate was shaken and incubated at room temperature for 30 min. The reaction was stopped by adding 50 µl of 0.5 M $H_2SO_4$ to each well and the absorbance read at 450 nm within 20 min of termination of the reaction.

# Appendix B.3.

**B.3.1. Results of one-way analysis of variance (ANOVA) of IFN-γ data obtained in blood samples of 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis*, 14 infected-unvaccinated sheep and 13 uninfected-unvaccinated control sheep.**

| Raw data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 27 | | | |
| F | 14.04 | | | |
| **R squared** | **0.5384** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 957.8 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 56.94 | 26 | 2.19 | |
| Residual (within columns) | 48.81 | 313 | 0.156 | |
| Total | 105.8 | 339 | | |

| ln(Y+1) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 27 | | | |
| F | 18.03 | | | |
| **R squared** | **0.5996** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 733.4 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 21.05 | 26 | 0.8094 | |
| Residual (within columns) | 14.05 | 313 | 0.0449 | |
| Total | 35.1 | 339 | | |
| **Tukey's Multiple Comparison Test** | Mean Diff. | q | P value | 95% CI of diff |
| 0v vs 0c | -0.0061 | 0.1057 | P > 0.05 | -0.3146 to 0.3024 |
| 2v vs 2c | -0.004504 | 0.07805 | P > 0.05 | -0.3130 to 0.3040 |
| 5v vs 5i | 0.1979 | 3.495 | P > 0.05 | -0.1048 to 0.5007 |
| 5v vs 5c | 0.2154 | 3.733 | P > 0.05 | -0.09311 to 0.5239 |
| **9v vs 9c** | 0.8117 | 13.45 | **P < 0.001** | 0.4890 to 1.134 |
| **18v vs 18c** | 0.6435 | 10.66 | **P < 0.001** | 0.3208 to 0.9663 |
| **27v vs 27c** | 0.567 | 9.139 | **P < 0.001** | 0.2353 to 0.8986 |
| **36v vs 36c** | 0.5903 | 9.515 | **P < 0.001** | 0.2586 to 0.9219 |
| **44v vs 44c** | 0.5015 | 7.817 | **P < 0.001** | 0.1586 to 0.8445 |
| 53v vs 53c | 0.2621 | 4.085 | P > 0.05 | -0.08088 to 0.6051 |
| 0i vs 0c | 0.007666 | 0.1328 | P > 0.05 | -0.3009 to 0.3162 |
| 2i vs 2c | -0.0001866 | 0.003233 | P > 0.05 | -0.3087 to 0.3083 |
| 5i vs 5c | 0.01751 | 0.3033 | P > 0.05 | -0.2910 to 0.3260 |
| 9i vs 9c | 0.04335 | 0.718 | P > 0.05 | -0.2794 to 0.3661 |
| **18i vs 18c** | 0.3523 | 5.836 | **P < 0.05** | 0.02959 to 0.6751 |
| 27i vs 27c | 0.204 | 3.288 | P > 0.05 | -0.1277 to 0.5356 |
| 36i vs 36c | 0.3112 | 4.937 | P > 0.05 | -0.02574 to 0.6481 |
| 44i vs 44c | 0.3048 | 4.655 | P > 0.05 | -0.04522 to 0.6547 |
| 53i vs 53c | 0.2038 | 2.96 | P > 0.05 | -0.1643 to 0.5718 |
| 0v vs 0i | -0.01377 | 0.2431 | P > 0.05 | -0.3165 to 0.2890 |
| 2v vs 2i | -0.004317 | 0.07624 | P > 0.05 | -0.3071 to 0.2984 |
| **9v vs 9i** | 0.7684 | 13.57 | **P < 0.001** | 0.4656 to 1.071 |
| 18v vs 18i | 0.2912 | 5.142 | P > 0.05 | -0.01156 to 0.5939 |
| **27v vs 27i** | 0.363 | 6.41 | **P < 0.01** | 0.06024 to 0.6657 |
| 36v vs 36i | 0.2791 | 4.836 | P > 0.05 | -0.02943 to 0.5876 |
| 44v vs 44i | 0.1968 | 3.146 | P > 0.05 | -0.1376 to 0.5311 |
| 53v vs 53i | 0.05832 | 0.8826 | P > 0.05 | -0.2949 to 0.4115 |

**i**-infected-unvaccinated sheep
**v**-infected-vaccinated sheep
**c**-control sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.2. Results of one-way analysis of variance (ANOVA) of antibody data obtained in serum samples of 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis*, 14 infected-unvaccinated sheep and 13 uninfected-unvaccinated control sheep.**

| Raw data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 27 | | | |
| F | 34.23 | | | |
| **R squared** | **0.7398** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 1255 | | | |
| ANOVA Table | **SS** | **df** | **MS** | |
| Treatment (between columns) | 144.4 | 26 | 5.555 | |
| Residual (within columns) | 50.8 | 313 | 0.1623 | |
| Total | 195.2 | 339 | | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 27 | | | |
| F | 100.2 | | | |
| **R squared** | **0.8927** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 268.6 | | | |
| ANOVA Table | **SS** | **df** | **MS** | |
| Treatment (between columns) | 498.5 | 26 | 19.17 | |
| Residual (within columns) | 59.92 | 313 | 0.1914 | |
| Total | 558.5 | 339 | | |
| **Tukey's Multiple Comparison Test** | Mean Diff. | q | P value | 95% CI of diff |
| 0i vs 0v | -0.03267 | 0.2794 | P > 0.05 | -0.6578 to 0.5925 |
| 2i vs 2v | -0.07652 | 0.6544 | P > 0.05 | -0.7017 to 0.5486 |
| 5i vs 5v | -1.174 | 10.04 | **P < 0.001** | -1.799 to -0.5488 |
| 9i vs 9v | -2.623 | 22.43 | **P < 0.001** | -3.248 to -1.998 |
| 18i vs 18v | -2.843 | 24.32 | **P < 0.001** | -3.469 to -2.218 |
| 27i vs 27v | -2.576 | 22.03 | **P < 0.001** | -3.201 to -1.951 |
| 36i vs 36v | -2.298 | 19.28 | **P < 0.001** | -2.935 to -1.661 |
| 44i vs 44v | -2.421 | 18.74 | **P < 0.001** | -3.111 to -1.730 |
| 53i vs 53v | -2.553 | 18.72 | **P < 0.001** | -3.283 to -1.824 |
| | | | | |
| 0i vs 0c | -0.03298 | 0.2767 | P > 0.05 | -0.6700 to 0.6041 |
| 2i vs 2c | -0.07772 | 0.6522 | P > 0.05 | -0.7148 to 0.5593 |
| 5i vs 5c | 0.04851 | 0.4071 | P > 0.05 | -0.5885 to 0.6856 |
| 9i vs 9c | -0.03295 | 0.2643 | P > 0.05 | -0.6994 to 0.6335 |
| 18i vs 18c | 0.2045 | 1.64 | P > 0.05 | -0.4619 to 0.8709 |
| 27i vs 27c | 0.5043 | 3.937 | P > 0.05 | -0.1805 to 1.189 |
| 36i vs 36c | 0.6055 | 4.653 | P > 0.05 | -0.09017 to 1.301 |
| 44i vs 44c | 0.5397 | 3.992 | P > 0.05 | -0.1830 to 1.262 |
| 53i vs 53c | 0.3813 | 2.682 | P > 0.05 | -0.3787 to 1.141 |
| | | | | |
| 0c vs 0v | 0.0003016 | 0.002531 | P > 0.05 | -0.6368 to 0.6374 |
| 2c vs 2v | 0.001192 | 0.01 | P > 0.05 | -0.6359 to 0.6382 |
| **5c vs 5v** | -1.222 | 10.26 | **P < 0.001** | -1.860 to -0.5854 |
| **9c vs 9v** | -2.59 | 20.78 | **P < 0.001** | -3.257 to -1.924 |
| **18c vs 18v** | -3.048 | 24.45 | **P < 0.001** | -3.714 to -2.381 |
| **27c vs 27v** | -3.08 | 24.05 | **P < 0.001** | -3.765 to -2.396 |
| **36c vs 36v** | -2.903 | 22.67 | **P < 0.001** | -3.588 to -2.219 |
| **44c vs 44v** | -2.96 | 22.35 | **P < 0.001** | -3.668 to -2.252 |
| **53c vs 53v** | -2.935 | 22.15 | **P < 0.001** | -3.643 to -2.226 |

**i**-infected-unvaccinated sheep
**v**-infected-vaccinated sheep
**c**-control sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.3. Results of one-way analysis of variance (ANOVA) of IFN-γ data obtained in blood samples of 7 infected-unvaccinated sheep in which AFO's were detected (AFO+ve), 7 infected-unvaccinated sheep in which AFO's were not detected (AFO-ve), and 13 untreated control sheep.**

| Raw data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 27 | | | |
| F | 8.775 | | | |
| **R squared** | **0.5443** | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 7.584 | 26 | 0.2917 | |
| Residual (within columns) | 6.349 | 191 | 0.03324 | |
| Total | 13.93 | 217 | | |

| ln(Y+1) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 27 | | | |
| F | 10.41 | | | |
| **R squared** | **0.5863** | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 4.178 | 26 | 0.1607 | |
| Residual (within columns) | 2.948 | 191 | 0.01543 | |
| Total | 7.126 | 217 | | |
| **Tukey's Multiple Comparison Test** | Mean Diff. | q | P value | 95% CI of diff |
| 0+ vs 0c | 0.01028 | 0.2497 | P > 0.05 | -0.2101 to 0.2307 |
| 2+ vs 27c | -0.009561 | 0.2209 | P > 0.05 | -0.2412 to 0.2221 |
| 5+ vs 5c | 0.0002682 | 0.006513 | P > 0.05 | -0.2201 to 0.2207 |
| 9+ vs 9c | 0.007475 | 0.176 | P > 0.05 | -0.2198 to 0.2348 |
| 18+ vs 18c | 0.2177 | 5.126 | P > 0.05 | -0.009554 to 0.4450 |
| 27+ vs 27c | 0.1198 | 2.767 | P > 0.05 | -0.1119 to 0.3515 |
| 36+ vs 36c | 0.1789 | 3.944 | P > 0.05 | -0.06386 to 0.4217 |
| 44+ vs 44c | 0.2979 | 6.19 | **P < 0.01** | 0.04038 to 0.5554 |
| 53+ vs 53c | 0.2793 | 4.83 | P > 0.05 | -0.03017 to 0.5888 |
| | | | | |
| 0+ vs 0- | 0.005237 | 0.1115 | P > 0.05 | -0.2460 to 0.2565 |
| 2+ vs 2- | -0.0177 | 0.3768 | P > 0.05 | -0.2690 to 0.2336 |
| 5+ vs 5- | -0.03448 | 0.7342 | P > 0.05 | -0.2858 to 0.2168 |
| 9+ vs 9- | -0.07174 | 1.528 | P > 0.05 | -0.3230 to 0.1795 |
| 18+ vs 18- | -0.2692 | 5.732 | **P < 0.05** | -0.5205 to -0.01789 |
| 27+ vs 27- | -0.1684 | 3.586 | P > 0.05 | -0.4197 to 0.08288 |
| 36+ vs 36- | -0.2457 | 5.026 | P > 0.05 | -0.5072 to 0.01589 |
| 44+ vs 44- | -0.01265 | 0.2378 | P > 0.05 | -0.2973 to 0.2720 |
| 53+ vs 53- | 0.1133 | 1.824 | P > 0.05 | -0.2191 to 0.4457 |
| | | | | |
| 0- vs 0c | 0.005047 | 0.1226 | P > 0.05 | -0.2153 to 0.2254 |
| 2- vs 2c | 0.008661 | 0.2103 | P > 0.05 | -0.2117 to 0.2291 |
| 2- vs 5c | 0.01694 | 0.4114 | P > 0.05 | -0.2034 to 0.2373 |
| 5- vs 5c | 0.03474 | 0.8436 | P > 0.05 | -0.1856 to 0.2551 |
| 9- vs 9c | 0.07922 | 1.865 | P > 0.05 | -0.1481 to 0.3065 |
| 18- vs 18c | 0.4869 | 11.46 | **P < 0.001** | 0.2596 to 0.7142 |
| 27- vs 27c | 0.2882 | 6.657 | **P < 0.01** | 0.05651 to 0.5199 |
| 36- vs 36c | 0.4246 | 9.807 | **P < 0.001** | 0.1929 to 0.6562 |
| 44- vs 44c | 0.3105 | 6.845 | **P < 0.01** | 0.06775 to 0.5533 |
| 53- vs 53c | 0.166 | 3.66 | P > 0.05 | -0.07674 to 0.4088 |

\+ AFO's positive infected-unvaccinated sheep
\- AFO's negative infected-unvaccinated sheep
**c** control sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.4. Results of Kruskal-Wallis test (non-parametric ANOVA) on antibody data obtained in serum samples of 7 infected-unvaccinated sheep in which AFO's were detected (AFO+ve), 7 infected-unvaccinated sheep in which AFO's were not detected (AFO-ve), and 13 untreated control sheep.**

| Raw data | | |
|---|---|---|
| **Kruskal-Wallis test** | | |
| P value | P<0.0001 | |
| Number of groups | 27 | |
| Kruskal-Wallis statistic | 95.97 | |
| | | |
| **Dunn's Multiple Comparison** | Difference in rank sum | P value |
| 0- vs 0c | -33.32 | P > 0.05 |
| 2- vs 2c | -38.65 | P > 0.05 |
| 5- vs 5c | -11.86 | P > 0.05 |
| 9- vs 9c | -41.63 | P > 0.05 |
| 18- vs 18c | 53.59 | P > 0.05 |
| 27- vs 27c | 75.03 | P > 0.05 |
| 36- vs 36c | 71.37 | P > 0.05 |
| 44- vs 44c | 76.13 | P > 0.05 |
| 53- vs 53c | 67.2 | P > 0.05 |
| | | |
| 0- vs 0+ | -39.5 | P > 0.05 |
| 2- vs 2+ | -27.29 | P > 0.05 |
| 5- vs 5+ | -23.21 | P > 0.05 |
| 9- vs 9+ | -54.29 | P > 0.05 |
| 18- vs 18+ | -6.214 | P > 0.05 |
| 27- vs 27+ | 13.36 | P > 0.05 |
| 36- vs 36+ | 17.9 | P > 0.05 |
| 44- vs 44+ | 10.08 | P > 0.05 |
| 53- vs 53+ | -2.417 | P > 0.05 |
| | | |
| 0+ vs 0c | 6.181 | P > 0.05 |
| 2+ vs 2c | -11.37 | P > 0.05 |
| 5+ vs 5c | 11.36 | P > 0.05 |
| 9+ vs 9c | 12.66 | P > 0.05 |
| 18+ vs 18c | 59.81 | P > 0.05 |
| 27+ vs 27c | 61.67 | P > 0.05 |
| 36+ vs 36c | 53.47 | P > 0.05 |
| 44+ vs 44c | 66.05 | P > 0.05 |
| 53+ vs 53c | 69.62 | P > 0.05 |

**+** AFO's positive infected-unvaccinated sheep
**-** AFO's negative infected-unvaccinated sheep
**c** control sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.5. Results of one-way analysis of variance (ANOVA) of weight data obtained in 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis* and 14 infected-unvaccinated sheep.**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 43.13 | | |
| **R squared** | **0.7684** | | |
| Bartlett's test for equal variances | | | |
| **Bartlett's statistic (corrected)** | 86.18 | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 20090 | 15 | 1339 |
| Residual (within columns) | 6054 | 195 | 31.05 |
| Total | 26140 | 210 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 66.25 | | | |
| **R squared** | **0.836** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 19.88 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 43.62 | 15 | 2.908 | |
| Residual (within columns) | 8.56 | 195 | 0.0439 | |
| Total | 52.18 | 210 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | P value | 95% CI of diff |
| -2v vs -2i | -0.04879 | 0.8714 | P > 0.05 | -0.3258 to 0.2282 |
| 2v vs 2i | -0.01053 | 0.188 | P > 0.05 | -0.2875 to 0.2665 |
| 9v vs 9i | -0.05996 | 1.071 | P > 0.05 | -0.3370 to 0.2171 |
| 18v vs 18i | -0.01996 | 0.3565 | P > 0.05 | -0.2970 to 0.2571 |
| 27v vs 27i | -0.01812 | 0.3235 | P > 0.05 | -0.2951 to 0.2589 |
| 36v vs 36i | -0.06892 | 1.208 | P > 0.05 | -0.3512 to 0.2134 |
| 44v vs 44i | 0.0715 | 1.156 | P > 0.05 | -0.2344 to 0.3774 |
| 53v vs 53i | 0.05861 | 0.8972 | P > 0.05 | -0.2646 to 0.3818 |

**i**-infected-unvaccinated sheep
**v**-infected-vaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.6. Results of Kruskal-Wallis test (non-parametric ANOVA) on growth rate data obtained in 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis* and 14 infected-unvaccinated sheep.**

| Raw data | | | |
|---|---|---|---|
| **Kruskal-Wallis test** | | | |
| P value | P<0.0001 | (Gaussian Approximation) | |
| Number of groups | 14 | | |
| Kruskal-Wallis statistic | 97.04 | | |
| **Dunn's Multiple Comparison Test** | | | |
| | Difference in rank | P value | Summary |
| 2v vs 2i | -2.821 | P> 0.05 | ns |
| 9v vs 9i | -18.18 | P > 0.05 | ns |
| 18v vs 18i | 8.964 | P > 0.05 | ns |
| 27v vs 27i | -1.5 | P > 0.05 | ns |
| 36v vs 36i | -11.13 | P > 0.05 | ns |
| 44v vs 44i | 23.47 | P > 0.05 | ns |
| 53v vs 53i | 38.49 | P > 0.05 | ns |

**i**-infected-unvaccinated sheep
**v**-infected-vaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.7. Results of Kruskal-Wallis test (non-parametric ANOVA) on faecal egg count data obtained in 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis* and 14 infected-unvaccinated sheep.**

| Raw data | | | |
|---|---|---|---|
| **Kruskal-Wallis test** | | | |
| P value | 0.0031 | (Gaussian Approximation) | |
| Number of groups | 14 | | |
| Kruskal-Wallis statistic | 31.21 | | |
| **Dunn's Multiple Comparison Test** | | | |
| | Difference in rank | P value | Summary |
| 2v vs 2i | 22.63 | P > 0.05 | ns |
| 9v vs 9i | 11.82 | P > 0.05 | ns |
| 18v vs 18i | -20.88 | P > 0.05 | ns |
| 27v vs 27i | -15.61 | P > 0.05 | ns |
| 36v vs 36i | -2.453 | P > 0.05 | ns |
| 44v vs 44i | -12.23 | P > 0.05 | ns |
| 53v vs 53i | -10.94 | P > 0.05 | ns |

i-infected-unvaccinated sheep
v-infected-vaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.8. Results of one-way analysis of variance (ANOVA) of erythrocyte count data obtained in 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis* and 14 infected-unvaccinated sheep.**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 20.9 | | |
| **R squared** | **0.6202** | | |
| Bartlett's test for equal variances | | | |
| **Bartlett's statistic (corrected)** | 47.39 | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 508.4 | 15 | 33.89 |
| Residual (within columns) | 311.4 | 192 | 1.622 |
| Total | 819.8 | 207 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 20.74 | | | |
| **R squared** | **0.6183** | | | |
| Bartlett's test for equal variances | | | | |
| Bartlett's statistic (corrected) | 43.34 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 3.443 | 15 | 0.2295 | |
| Residual (within columns) | 2.125 | 192 | 0.01107 | |
| Total | 5.569 | 207 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | P value | 95% CI of diff |
| 2v vs 2i | -0.0738 | 2.575 | P > 0.05 | -0.2156 to 0.06797 |
| 5v vs 5i | -0.07068 | 2.514 | P > 0.05 | -0.2098 to 0.06844 |
| **9v vs 9i** | -0.1589 | 5.651 | **P < 0.05** | -0.2980 to -0.01978 |
| 18v vs 18i | -0.06124 | 2.137 | P > 0.05 | -0.2030 to 0.08053 |
| 27v vs 27i | -0.02553 | 0.891 | P > 0.05 | -0.1673 to 0.1162 |
| 36v vs 36i | -0.04504 | 1.572 | P > 0.05 | -0.1868 to 0.09673 |
| 44v vs 44i | -0.03469 | 1.117 | P > 0.05 | -0.1883 to 0.1189 |
| 53v vs 53i | -0.05014 | 1.528 | P > 0.05 | -0.2124 to 0.1122 |

i-infected-unvaccinated sheep
v-infected-vaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.9. Results of one-way analysis of variance (ANOVA) of haemoglobin data obtained in 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis* and 14 infected-unvaccinated sheep.**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 24.24 | | |
| **R squared** | **0.6545** | | |
| Bartlett's test for equal variances | | | |
| **Bartlett's statistic (corrected)** | 65.41 | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 56870 | 15 | 3791 |
| Residual (within columns) | 30020 | 192 | 156.4 |
| Total | 86890 | 207 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 22.72 | | | |
| **R squared** | **0.6396** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 59.32 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 3.212 | 15 | 0.2142 | |
| Residual (within columns) | 1.81 | 192 | 0.009426 | |
| Total | 5.022 | 207 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | P value | 95% CI of diff |
| 2v vs 2i | -0.04893 | 1.85 | P > 0.05 | -0.1797 to 0.08189 |
| 5v vs 5i | -0.03979 | 1.534 | P > 0.05 | -0.1682 to 0.08858 |
| **9v vs 9i** | -0.1292 | 4.981 | **P < 0.05** | -0.2576 to -0.0008596 |
| 18v vs 18i | -0.005788 | 0.2189 | P > 0.05 | -0.1366 to 0.1250 |
| 27v vs 27i | 0.02068 | 0.782 | P > 0.05 | -0.1101 to 0.1515 |
| 36v vs 36i | -0.0006018 | 0.02276 | P > 0.05 | -0.1314 to 0.1302 |
| 44v vs 44i | 0.02113 | 0.7375 | P > 0.05 | -0.1206 to 0.1629 |
| 53v vs 53i | 0.01509 | 0.4986 | P > 0.05 | -0.1347 to 0.1649 |

**i**-infected-unvaccinated sheep
**v**-infected-vaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.10. Results of one-way analysis of variance (ANOVA) of total leukocyte data obtained in 14 sheep vaccinated 2 weeks after oral infection with *M. paratuberculosis* and 14 infected-unvaccinated sheep.**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 14.16 | | |
| **R squared** | **0.5253** | | |
| Bartlett's test for equal variances | | | |
| **Bartlett's statistic (corrected)** | 37.56 | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 1577 | 15 | 105.1 |
| Residual (within columns) | 1425 | 192 | 7.421 |
| Total | 3001 | 207 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 16.58 | | | |
| **R squared** | **0.5643** | | | |
| Bartlett's test for equal variances | | | | |
| **Bartlett's statistic (corrected)** | 32.28 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 23.77 | 15 | 1.585 | |
| Residual (within columns) | 18.36 | 192 | 0.0956 | |
| Total | 42.13 | 207 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | P value | 95% CI of diff |
| 2v vs 2i | 0.01002 | 0.119 | P > 0.05 | -0.4066 to 0.4266 |
| 5v vs 5i | 0.002299 | 0.02782 | P > 0.05 | -0.4065 to 0.4111 |
| 9v vs 9i | -0.1318 | 1.595 | P > 0.05 | -0.5406 to 0.2771 |
| 18v vs 18i | -0.03452 | 0.4099 | P > 0.05 | -0.4511 to 0.3821 |
| 27v vs 27i | -0.0382 | 0.4536 | P > 0.05 | -0.4548 to 0.3784 |
| 36v vs 36i | 0.08092 | 0.9609 | P > 0.05 | -0.3357 to 0.4975 |
| 44v vs 44i | 0.1314 | 1.439 | P > 0.05 | -0.3202 to 0.5829 |
| 53v vs 53i | -0.2449 | 2.54 | P > 0.05 | -0.7218 to 0.2321 |

**i**-infected-unvaccinated sheep
**v**-infected-vaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.11. Results of one-way analysis of variance (ANOVA) of erythrocyte count data obtained in 7 infected-unvaccinated sheep in which AFO's were detected (AFO+ve) and 7 infected-unvaccinated sheep in which AFO's were not detected (AFO-ve).**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 10.01 | | |
| **R squared** | **0.6332** | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 313.8 | 15 | 20.92 |
| Residual (within columns) | 181.8 | 87 | 2.09 |
| Total | 495.6 | 102 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 10.21 | | | |
| **R squared** | **0.6376** | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 2.015 | 15 | 0.1343 | |
| Residual (within columns) | 1.145 | 87 | 0.01316 | |
| Total | 3.16 | 102 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | P value | 95% CI of diff |
| 2- vs 2+ | 0.02828 | 0.6521 | P > 0.05 | -0.1889 to 0.2455 |
| 5- vs 5+ | 0.0225 | 0.5189 | P > 0.05 | -0.1947 to 0.2397 |
| 9- vs 9+ | 0.1543 | 3.558 | P > 0.05 | -0.06291 to 0.3714 |
| 18- vs 18+ | 0.0887 | 2.046 | P > 0.05 | -0.1285 to 0.3059 |
| 27- vs 27+ | 0.05055 | 1.166 | P > 0.05 | -0.1666 to 0.2677 |
| 36- vs 36+ | 0.00191 | 0.04232 | P > 0.05 | -0.2241 to 0.2280 |
| 44- vs 44+ | -0.03882 | 0.7903 | P > 0.05 | -0.2848 to 0.2072 |
| 53- vs 53+ | 0.004607 | 0.08032 | P > 0.05 | -0.2827 to 0.2919 |

+ AFO's positive infected-unvaccinated sheep
- AFO's negative infected-unvaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.12. Results of one-way analysis of variance (ANOVA) of haemoglobin data obtained in 7 infected-unvaccinated sheep in which AFO's were detected (AFO+ve) and 7 infected-unvaccinated sheep in which AFO's were not detected (AFO-ve).**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 13.19 | | |
| **R squared** | **0.6946** | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 37910 | 15 | 2527 |
| Residual (within columns) | 16670 | 87 | 191.6 |
| Total | 54570 | 102 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| P value | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 12.99 | | | |
| **R squared** | **0.6913** | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 2.087 | 15 | 0.1391 | |
| Residual (within columns) | 0.9318 | 87 | 0.01071 | |
| Total | 3.019 | 102 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | P value | 95% CI of diff |
| 2+ vs 2- | -0.009967 | 0.2548 | P > 0.05 | -0.2059 to 0.1859 |
| 5+ vs 5- | -0.01525 | 0.3898 | P > 0.05 | -0.2112 to 0.1807 |
| 9+ vs 9- | -0.1642 | 4.197 | P > 0.05 | -0.3601 to 0.03175 |
| 18+ vs 18- | -0.07662 | 1.959 | P > 0.05 | -0.2725 to 0.1193 |
| 27+ vs 27- | -0.05374 | 1.374 | P > 0.05 | -0.2496 to 0.1422 |
| 36+ vs 36- | -0.01391 | 0.3416 | P > 0.05 | -0.2178 to 0.1900 |
| 44+ vs 44- | 0.01183 | 0.2671 | P > 0.05 | -0.2101 to 0.2338 |
| 53+ vs 53- | -0.07698 | 1.488 | P > 0.05 | -0.3361 to 0.1822 |

+ AFO's positive infected-unvaccinated sheep
- AFO's negative infected-unvaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

**B.3.13. Results of one-way analysis of variance (ANOVA) of total leukocyte data obtained in 7 infected-unvaccinated sheep in which AFO's were detected (AFO+ve) and 7 infected-unvaccinated sheep in which AFO's were not detected (AFO-ve).**

| Raw data | | | |
|---|---|---|---|
| **One-way analysis of variance** | | | |
| P value | P<0.0001 | | |
| Number of groups | 16 | | |
| F | 6.135 | | |
| **R squared** | **0.514** | | |
| ANOVA Table | SS | df | MS |
| Treatment (between columns) | 889.1 | 15 | 59.28 |
| Residual (within columns) | 840.6 | 87 | 9.662 |
| Total | 1730 | 102 | |

| ln(Y) transformed data | | | | |
|---|---|---|---|---|
| **One-way analysis of variance** | | | | |
| Pvalue | P<0.0001 | | | |
| Number of groups | 16 | | | |
| F | 7.405 | | | |
| **R squared** | 0.5608 | | | |
| ANOVA Table | SS | df | MS | |
| Treatment (between columns) | 12.84 | 15 | 0.8562 | |
| Residual (within columns) | 10.06 | 87 | 0.1156 | |
| Total | 22.9 | 102 | | |
| **Tukey's Multiple Comparison Test** | | | | |
| | Mean Diff. | q | Pvalue | 95% CI of diff |
| 2- vs 2+ | 0.2224 | 1.731 | P > 0.05 | -0.4213 to 0.8662 |
| 5- vs 5+ | 0.09022 | 0.702 | P > 0.05 | -0.5535 to 0.7339 |
| 9- vs 9+ | -0.2836 | 2.206 | P > 0.05 | -0.9273 to 0.3602 |
| 18- vs 18+ | -0.1701 | 1.323 | P > 0.05 | -0.8138 to 0.4736 |
| 27- vs 27+ | -0.1796 | 1.397 | P > 0.05 | -0.8233 to 0.4641 |
| 36- vs 36+ | 0.09138 | 0.6831 | P > 0.05 | -0.5786 to 0.7614 |
| 44- vs 44+ | 0.07413 | 0.5091 | P > 0.05 | -0.6551 to 0.8033 |
| 53- vs 53+ | 0.0797 | 0.4688 | P > 0.05 | -0.7718 to 0.9312 |

\+ AFO's positive infected-unvaccinated sheep
\- AFO's negative infected-unvaccinated sheep
numbers indicate weeks after oral inoculation with *M. paratuberculosis*

# Appendix B. 4.

**Cause and time of death of 3 uninfected and unvaccinated (control) sheep**

| Sheep ID | Time of death* | Cause of death | Histological evidence of paratuberculosis |
|---|---|---|---|
| **35** | 7 | asphixiation due to entangling in a fence | nd |
| **62** | 8 | internal injuries after being run over by a motorbike | nd |
| **48** | 34 | haemorrhagic shock due to hepatic rupture | - |

***** weeks after initiation of the experiment
**nd** not determined (animal not delivered for post-mortem examinations)
**-** negative

# Appendix B. 5.

**Results of the PCR assay on duplicate samples of ileal and ileocaecal lymph node DNA from 14 sheep infected experimentally with *M. paratuberculosis*, 14 sheep vaccinated against paratuberculosis 2 weeks after infection and 13 uninfected and unvaccinated controls compared with results obtained by histology.**

| | Sheep ID | Tissues examined | | | | | |
|---|---|---|---|---|---|---|---|
| | | jejunum | ileum | | caudal mesenteric lymph node | ileocaecal lymph node | |
| | | Histology results | Histology results | PCR results | Histology results | Histology results | PCR results |
| Control | 31 | - | - | - | - | - | - |
| | 35 | nd | nd | nd | nd | nd | nd |
| | 37 | - | - | - | - | - | - |
| | 38 | - | - | - | - | - | - |
| | 39 | - | - | - | - | - | - |
| | 41 | - | - | - | - | - | - |
| | 42 | - | - | - | - | - | - |
| | 45 | - | - | - | - | - | - |
| | 48 | - | - | - | - | - | - |
| | 50 | - | - | - | - | - | - |
| | 59 | - | - | - | - | - | - |
| | 62 | nd | nd | nd | nd | nd | nd |
| | 63 | - | - | - | - | - | - |
| Unvccinated | 32 | - | - | - | (+/-)* | - | - |
| | 34 | + | + | + | + | + | - |
| | 36 | + | + | + | + | + | + |
| | 49 | (+/-) | - | - | - | - | - |
| | 51 | +/- | +/-* | - | +/- | +/- | + |
| | 52 | - | - | - | - | - | - |
| | 55 | + | + | + | + | + | + |
| | 56 | - | - | - | - | - | - |
| | 57 | - | - | - | - | - | - |
| | 60 | + | + | + | + | + | + |
| | 61 | + | + | + | +/- | +/- | + |
| | 67 | +/- | +/- | + | +/- | (+/-)* | + |
| | 68 | + | + | + | + | +/- | +/- |
| | 69 | - | + | + | | + | + |
| Vaccinated | 33 | - | - | - | - | - | - |
| | 40 | - | - | - | - | +/-* | - |
| | 43 | +/-* | +/-* | - | +/-* | +/- | + |
| | 44 | - | (+/-)* | - | - | - | - |
| | 46 | +/- | +/- | + | +/- | +/- | + |
| | 47 | (+/-) | - | - | - | - | - |
| | 53 | - | - | - | +/- | +/- | - |
| | 54 | - | - | - | - | - | - |
| | 58 | - | - | - | - | - | +/- |
| | 64 | +/- | +/- | + | +/- | +/- | - |
| | 65 | (+/-)* | - | - | (+/-)* | +/-* | - |
| | 66 | - | - | - | - | - | - |
| | 70 | - | - | - | - | - | - |
| | 71 | - | - | - | - | - | - |

\+ presence of 400-bp band on the gel and a signal present on the dot blot / granulomatous lesions with AFO's (lepromatous morphology); +/- signal present on the dot blot but no visible 400-bp band on the gel / granulomatous lesions without AFO's (equivocal lesions of lepromatous morphology); (+/-) lesions showing central necrosis in which AFO's were not detected (equivocal lesions of tuberculoid morphology); - absence of 400-bp band on the gel and no signal on the dot blot / neither lepromatous- nor tuberculoid-like lesions detected (no AFO's)

* presence of multinucleate giant cells; **nd** not done

# Appendix C.1.

**Table C.1. Numbers of sheep detected by immunological tests in a group of 30 sheep inoculated orally with *M. paratuberculosis* in the first month of life (Trial 1) in relation to results derived from histology and PCR on samples of ileum and ileocaecal lymph node.**

| Category of sheep | Number of sheep identified as positive by immunological tests | | | | |
|---|---|---|---|---|---|
| | CFT | AGID | ELISA 0.1 cut-off | ELISA 0.05 cut-off | Combined ELISA 0.05 cut-off, AGID and CFT |
| A (n=14) | 12 | 12 | 12 | 14 | 14 |
| B (n=9) | 6 | 7 | 3 | 7 | 8 |
| C (n=4) | 1 | 0 | 2 | 3 | 3 |
| D (n=1) | 1 | 1 | 0 | 1 | 1 |
| E (n=2) | 2 | 2 | 2 | 2 | 2 |
| Total (n=30) | 22 | 22 | 19 | 27 | 28 |
| Fisher exact test P value (A vs B) | 0.3428 | 1 | 0.0228 | 0.1423 | nd |
| Relative Risk | 1.667 | 1.263 | 3.2 | na | nd |

**Category:**

**A** - sheep with paratuberculosis confirmed by histology and PCR

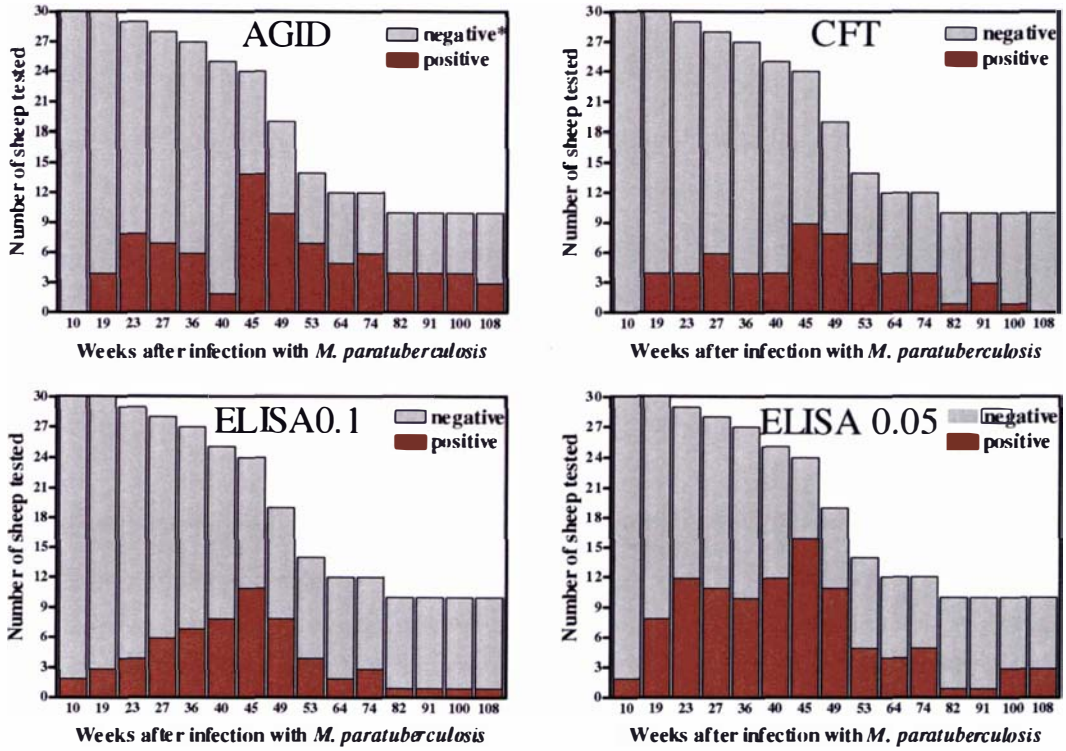
**B** - sheep that tested negative by PCR and had no histological evidence of paratuberculosis in tissues examined

**C** - sheep that tested positive by PCR but had equivocal lesions in tissues examined

**D** - sheep that gave suspicious results in PCR but had no histological evidence of paratuberculosis in tissues examined

**E** - sheep that were not available for histological examination but had acid fast organisms detected in faeces

**Figure C.1. Numbers of sheep tested positive by immunological tests at various sampling points in a group of 30 sheep inoculated orally with *M. paratuberculosis* in the first month of life (Trial 1).**



# Appendix C.2.

**Table C.2. Numbers of sheep detected by immunological tests in a group of 14 sheep inoculated orally with *M. paratuberculosis* in the second month of life (Trial 2) in relation to results derived from histology and PCR on samples of ileum and ileocaecal lymph node.**

| Category of sheep | Number of sheep identified as positive by immunological tests | | | | | |
|---|---|---|---|---|---|---|
| | CFT | AGID | ELISA 0.1 cut-off | ELISA 0.05 cut-off | Combined ELISA 0.05 cut-off, AGID and CFT | IFN |
| A (n=7) | 2 | 5 | 1 | 4 | 6 | 7 |
| B (n=5) | 4 | 5 | 3 | 4 | 5 | 5 |
| C (n=2) | 1 | 2 | 1 | 1 | 2 | 2 |
| Total (n=14) | 7 | 12 | 5 | 9 | 13 | 14 |
| Fisher exact test P value (A vs B) | 0.2424 | 0.4697 | 0.2222 | 0.5758 | nd | na |
| Relative Risk | 0.4 | 0.5 | 0.3333 | 0.3333 | nd | na |

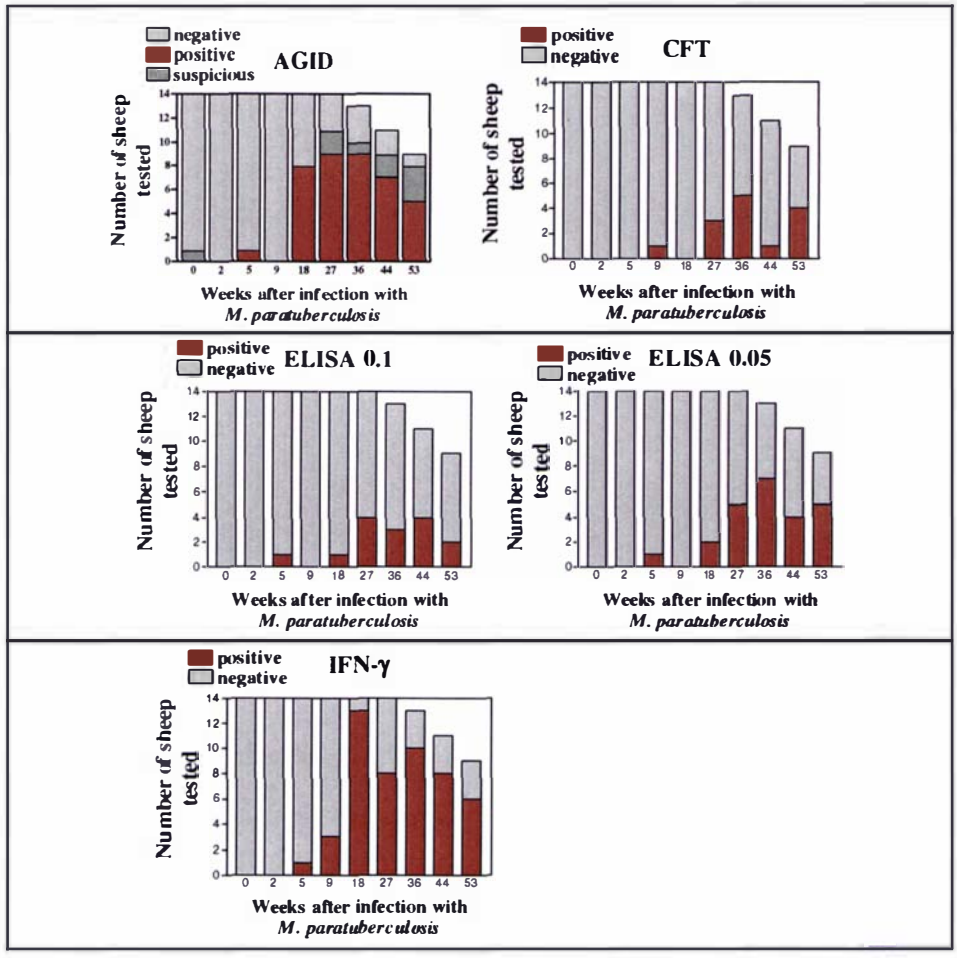
**Category:**

**A**-sheep with paratuberculosis confirmed by histology and PCR

**B**-sheep that tested negative by PCR and had no histological evidence of paratuberculosis in tissues examined,

**C**-sheep that tested positive by PCR but had equivocal lesions in tissues examined.

**Figure C.1. Numbers of sheep tested positive by immunological tests at various sampling points in a group of 14 sheep inoculated orally with *M. paratuberculosis* in the second month of life (Trial 2).**



# Appendix D

**Results of statistical analysis of data presented in Table 8.1.**

| Data analysed | Results of statistical anlysis (P value of Fisher exact test) | | |
|---|---|---|---|
| | Category of sheep | | |
| | A (n=10) | B (n=9) | Total (A+B) (n=19) |
| CFT vs AGID | >0.05 | >0.05 | >0.05 |
| CFT vs ELISA 0.1 cut-off | >0.05 | >0.05 | >0.05 |
| CFT vs ELISA 0.05 cut-off | >0.05 | >0.05 | >0.05 |
| AGID vs ELISA 0.1 cut-off | >0.05 | >0.05 | >0.05 |
| AGID vs ELISA 0.05 cut-off | >0.05 | >0.05 | >0.05 |
| Blood-IFN/ON vs CFT | >0.05 | >0.05 | >0.05 |
| Blood-IFN/ON vs AGID | >0.05 | >0.05 | >0.05 |
| Blood-IFN/ON vs ELISA 0.1 cut-off | >0.05 | >0.05 | >0.05 |
| Blood-IFN/ON vs ELISA 0.05 cut-off | >0.05 | >0.05 | >0.05 |
| Blood-IFN/48 hours vs CFT | >0.05 | >0.05 | >0.05 |
| Blood-IFN/48 hours vs AGID | >0.05 | >0.05 | >0.05 |
| Blood-IFN/48 hours vs ELISA 0.1 cut-off | >0.05 | >0.05 | >0.05 |
| Blood-IFN/48 hours vs ELISA 0.05 cut-off | >0.05 | >0.05 | >0.05 |
| PLN-IFN/ON vs CFT | **0.0001** | >0.05 | **0.0002** |
| PLN-IFN/ON vs AGID | **0.0198** | >0.05 | **0.017** |
| PLN-IFN/ON vs ELISA 0.1 cut-off | **0.0011** | **0.0152** | **<0.0001** |
| PLN-IFN/ON vs ELISA 0.05 cut-off | **0.0011** | >0.05 | **0.0025** |
| PLN-IFN/ON vs Blood-IFN/ON | **0.0055** | >0.05 | **0.0069** |
| PLN-IFN/ON vs Blood-IFN/48 hours | **0.0055** | >0.05 | **0.0069** |
| PLN-IFN/48 hours vs CFT | **0.0002** | **0.0294** | **<0.0001** |
| PLN-IFN/48 hours vs AGID | **0.03** | >0.05 | **0.0011** |
| PLN-IFN/48 hours vs ELISA 0.1 cut-off | **0.0019** | **0.0023** | **<0.0001** |
| PLN-IFN/48 hours vs ELISA 0.05 cut-off | **0.0019** | >0.05 | **0.0001** |
| PLN-IFN/48 hours vs Blood-IFN/ON | **0.0089** | >0.05 | **0.0004** |
| PLN-IFN/48 hours vs Blood-IFN hours | **0.0089** | >0.05 | **0.0004** |

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**Cogito ergo sum**

versus

**Sum ergo cogito**

## Publication(s) arising from this thesis

J.M.Gwozdz, M.P.Reichel, A.Murray, B.W.Manktelow, D.M.West, K.G.Thompson. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine tissues and blood by the polymerase chain reaction. Vet. Microbiol. 1997; 51:233-244.