IMMUNOLOGICAL AND MOLECULAR DISCRIMINATION OF *MYCOPLASMA BOVIS* FROM OTHER *MYCOPLASMA* SPECIES IN CATTLE

Thesis submitted

by

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ABSTRACT

Mycoplasma bovis is one of the most pathogenic bovine mycoplasmas causing extensive economic losses due to mastitis, pneumonia, keratoconjunctivitis, abortion and infertility. Current methodologies for detecting and identifying *Mycoplasma bovis* are time consuming and have poor sensitivity and specificity. The present studies were carried out to develop improved diagnostic techniques for the discrimination of *Mycoplasma bovis* from other *Mycoplasma* species and other bacterial species that cause infections in cattle. Four different detection techniques were developed and evaluated. These were isolation by conventional cultural methods, antigen and antibody detection by enzyme linked immunosorbent assay, development of a specific DNA probe and development of a polymerase chain reaction assay using primers specific for *Mycoplasma bovis.*

To evaluate cultural methods for the detection of *M. bovis*, milk samples and lung tissue specimens were obtained from the Atherton Tableland and a Townsville abattoir, respectively. A total of 500 lungs was examined for macroscopic evidence of pneumonia. Forty lungs had lesions macroscopically resembling *Mycoplasma* pneumonia. Fifteen were examined by histopathology and the microscopic lesions of interstitial pneumonia suggested that *Mycoplasma* was involved. *Mycoplasma bovis* was isolated from three cases suggesting a sensitivity for cultural methods of 20%. From the 92 individual quarter milk samples tested, two were positive by cultural methods. In the polymerase chain reaction developed, 35 of 92 quarters (38%) were positive for *Mycoplasma bovis*, again confirming the poor sensitivity of culture. Cultural methods were extremely insensitive and time consuming and commonly the cultures were overgrown by bacterial contaminants.

As an alternative to cultural methods, the detection of antibodies to *Mycoplasma bovis* and *Mycoplasma bovis* antigens in clinically diseased animals was attempted. An indirect enzyme linked immunosorbent assay was initially developed to detect the presence of *Mycoplasma bovis* antibody in animal and human sera that was used in the production of the *Mycoplasma* media used in these studies. The enzyme linked immunosorbent assay was also used to detect hybridomas secreting *Mycoplasma bovis* antibody during the production of monoclonal antibodies. It was found that under optimal conditions the indirect enzyme linked immunosorbent assay is fast and reproducible and a convenient and sensitive method for the detection of *Mycoplasma bovis* antibody in cattle sera. The limitations of the method include an extensive purification of antigen, and also some non-specific background signal. To improve antibody and antigen detection a panel of 15 monoclonal antibodies was produced using *Mycoplasma bovis* whole-cell antigen. Only one monoclonal antibody was specific to *Mycoplasma bovis*, while others were found to cross react with *Mycoplasma arginini, Mycoplasma agalactiae, Mycoplasma bovirhinis, Mycoplasma group 7, Mycoplasma bovigenitalium* and *Mycoplasma dispar.* An indirect enzyme linked immunosorbent assay using the specific

monoclonal antibody was useful for the clinical identification of *Mycoplasma bovis* isolated using cultural methods. Using this procedure, the three *Mycoplasma* isolated from the lung samples and the two *Mycoplasma* isolates from quarter milk samples were confirmed as a *Mycoplasma bovis.* This method was found to be difficult to standardise for the direct detection of *Mycoplasma* antigen in clinical samples, due to low sensitivity and specificity. A blocking ELISA was developed for the detection of specific *Mycoplasma bovis* antibody in serum. Using this method the specificity and sensitivity of the assay were improved and the background reaction was eliminated. This method revealed the existence of *Mycoplasma bovis* antibodies in 60 out of 100 (60%) serum samples from north Queensland dairy cattle. However the use of antibody detection by enzyme linked immunosorbent assay is limited by the fact that antibody titres become detectable only 10 to 14 days after the onset of disease. Because the sensitivity of the enzyme linked immunosorbent assay was not sufficient for reliable identification of sera containing antibodies to *Mycoplasma bovis*, a DNA probe for detection of *Mycoplasma bovis* was developed.

A genomic library was prepared from *Mycoplasma bovis* DNA digested with *Sau* 3AI and cloned into pUC19. Colony hybridisation, using a probe prepared from purified *Mycoplasma bovis* DNA, was used to identify colonies of interest. *Mycoplasma bovis* DNA fragments were retrieved from recombinant plasmids by digestion with *Eco*RI and *Hin*dIII. This DNA was used to prepare randomly primed probes for dot blot hybridisation analysis with immobilised DNA from two strains of *Mycoplasma bovis, Mycoplasma dispar, Mycoplasma agalactiae,* two strains of *Mycoplasma bovigenitalium, Mycoplasma ovipneumoniae,* a *Group 7* strain, *Mycoplasma arginini, Staphylococcus aureus, Staphylococcus* spp., *Streptococcus agalactiae, Streptococcus uberis, Corynebacterium bovis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pasturella* sp., *Brucella abortus, Yersinia enterocolitica, Enterobacter cloacae, Enterobacter faecalis, Serratia marcescens, Bacillus subtilis and Escherichia coli.* Four probes were found to hybridise only with *M. bovis* and *M. ovipneumoniae* genomic DNA, whereas one probe reacted only with genomic DNA from one of the two *Mycoplasma bovis* strains. Sensitivity of the dot blot hybridisation assay was 200 colony forming units per millilitre.

To increase the sensitivity of detection further, an *Mycoplasma bovis*-specific polymerase chain reaction assay was developed. The primers were designed using sequences obtained from the DNA probe which discriminated *Mycoplasma bovis* from all other *Mycoplasma* DNA tested. The minimum amount of target DNA that could be detected by the polymerase chain reaction assay was that isolated from 10-20 colony forming units per millilitre. The *Mycoplasma bovis*-specific polymerase chain reaction assay was therefore 10 times more sensitive than dot blot hybridisation. The identity of the three *Mycoplasma* spp. isolated from the cattle lung samples and the two isolates from the quarter milk samples (that showed evidence of clinical mastitis) were reconfirmed by using the polymerase

chain reaction assay. The assay was highly sensitive and specific for the detection of *Mycoplasma bovis* in milk samples, lungs and also nasal secretions.

The assay was applied for the first time to estimate the prevalence of *Mycoplasma bovis* infection in dairy cattle in Australia. In the first study, 186 and 165 bulk milk samples from north Queensland and Victoria respectively, were supplied from milk factories following routine somatic cell counting. Based on published guidelines for the interpretation of somatic cell count in bulk milk as an indicator of mastitis in a herd, five groups were determined: 1) slight $<250 \times 10^3$, 2) average $250\text{-}500 \times 10^3$, 3) above average $500\text{-}750 \times 10^3$, 4) bad $750\text{-}1000 \times 10^3$, 5) severe >1000. The association of *Mycoplasma bovis* infection in north Queensland milk samples with level of mastitis was: 32% of cases recognised as slight, 50% as average and in 65% of cases recognised as having above average level of mastitis. For milk samples from Victoria, *Mycoplasma bovis* was associated with 32% of cases recognised as having a slight level of mastitis, 71% average and 82% as having an above average level of mastitis.

In a study of 92 composite milk samples from individual cows in a single herd from Queensland, *Mycoplasma bovis* was detected in 55.5 % of cases recognised as having a slight level of mastitis, 70.5 % as average, 66.6% above average, 50% bad and 95% as having a severe level of mastitis.

A total of 52 quarter milk samples from cows with persistently high somatic cell counts in their composite milk were tested by *Mycoplasma bovis*-specific polymerase chain reaction, and culture and the association with major mastitis pathogens was investigated. *Mycoplasma bovis* was detected by polymerase chain reaction in 77% of samples tested of which 19% were infected with *M. bovis* alone, without any other bacteria detected, 17% had *Mycoplasma bovis* in combination with a major mastitis pathogen, and 40% had *Mycoplasma bovis* in combination with a non-major mastitis pathogen. A high association was found between positive reactors detected by indirect or blocking enzyme linked immunosorbent assay and animals found positive for *Mycoplasma bovis* by *Mycoplasma bovis*-specific polymerase chain reaction. The prevalence of *Mycoplasma bovis* in the respiratory tract of cattle and calves was also investigated using *Mycoplasma bovis*-specific polymerase chain ration and a close correlation between *Mycoplasma bovis* infection of the respiratory tract and the udder of cattle was found, suggesting the probable route in the infection cycle.

It is believed that *Mycoplasma bovis* infections are widespread in dairy cattle and has the potential to produce disease alone or to predispose the udder to disease caused by major mastitis pathogen and other environmental pathogens. These initial studies have revealed a previously unrecognised high prevalence of *Mycoplasma bovis* in dairy cattle in north Queensland and Victoria. These studies also give a clear association between *Mycoplasma bovis* and elevated somatic cell counts. The role of

Mycoplasma bovis as a cause of, or predisposing factor for bovine mastitis, is an area that requires further study.

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Ali GHADERSOHI
June 1997

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2YTA	2YT broth containing 150 µg/mL ampicillin
ABTS	2,2-Azino-di-[3-ethylbenzthizolin sulfonate (6)]
APS	Ammonium persulphate
BCA	Bicinchoninic acid
BMSCC	Bulk milk somatic cell count
BSA	Bovine serum albumin
CBPP	Contagious bovine pleuropneumoniae
CFT	Complement fixation test
CFU	Colony forming units
CIAP	Calf intestinal alkaline phosphatase
cps	Counts per second
DAB	Diaminobenzidine tetrahydrochloride dihydrate
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNPTs	Deoxynucleotide triphosphates
EDTA	Ethylene diaminine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FAB	Frey's arginine broth
FCS	Foetal calf serum
FMB	Frey's *Mycoplasma* broth
GTE	Glucose Tris EDTA
HAT	Hypoxanthine aminoptrin thymidine
HI	Haemagglutination inhibition
HPRT	Hypoxanthine phosphoribosyl transferase
HRPO	Horseradish peroxidase
HT	Hypoxanthine thymidine
IF	Immunofluoerescence
IHA	Indirect haemagglutination
IP	Intraperitoneally
IP	Immunoperoxidase
IPTG	Isopropylthio-β-D-galactoside
MA	*Mycoplasma* agar
MAb	Monoclonal antibody
MB	*Mycoplasma* broth
MB-PCR	*Mycoplasma bovis* polymerase chain reaction
MBM	*Mycoplasma* broth medium
MM	*Mycoplasma* medium
MMP	Major mastitis pathogen
MWM	Molecular weight marker
MWS	*Mycoplasma* wash solution
NAD	Nicotinamide adenine dinucleotide

NADH	Nicotinamide-adenine-dinucleotide-H^+
NBCS	New born calf serum
NRC	Non-reactive control serum
NTE	NaCl Tris EDTA
OD	Optical density
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline containing Tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Percentage inhibition
PMN	Polymorphonuclear neutrophil
PMSF	Phenyl methyl sulfonyl fluoride
PPLO	Pleuropneumonia-like organism
PRS	Polyclonal rabbit serum
QDHIC	Queensland Dairy Herd Improvement Centre
SCC	Somatic cell count
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TE	Tris EDTA
TEMED	Tetramethylenediamine
TEN-TC	Tris di-sodium ethylenediaminetetra-acetate sodium chloride Tween 20 casein
TK	Thymidine kinase
VsPs	Variable surface lipoproteins

PUBLICATIONS ARISING FROM THIS THESIS

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GHADERSOHI A, HIRST R G and FAYAZI Z (In preparation) Development of the monoclonal blocking ELISA for the detection of antibody to *Mycoplasma bovis* in cattle sera and comparison with *Mycoplasma bovis* PCR.

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CHAPTER 1

GENERAL INTRODUCTION

Mycoplasma bovis is one of the most pathogenic bovine *Mycoplasma* species. It is considered associated mainly with mastitis but also arthritis, pneumonia and genital disorders in bulls and cows (Jasper 1981; Royan et al. 1983; Kissi et al. 1985; Bocklisch et al. 1986; Howard et al. 1987; Jasper et al. 1987; Gonzalez et al. 1992). *Mycoplasma bovis* has been reported with high frequency worldwide (Erno and Perreau 1985; Ter-laak et al. 1992b). The organism was first isolated from a mastitis case in 1961 by Hale et al. (1962) and called *M. agalactiae* var. *bovis.* It was classified subsequently as a separate species, *Mycoplasma bovis,* based on its phenotypic characterisation and by nucleic acid hybridisation (Askaa and Erno 1976). Although isolation of *M. bovis* associated with mastitis was first reported by Cottew in 1970, since then there have been very few reports on the isolation of *Mycoplasma* from, or recognition of the disease, in dairy cattle in Australia.

The mechanisms by which this organism causes damage are thought to be principally through attachment to host cells (Thomas et al. 1991; Sachse et al. 1993b), cell injury from production of toxic substances such as a complex polysaccharide (Geary et al. 1981), deprivation of nutrients from, or alteration of, cell components and disruption of host cell metabolism (Simecka et al. 1992). The mechanisms of entry of *M. bovis* into the host cell, may be similar to that of *Mycoplasma penetrans*, *Mycoplasma pneumoniae* and *Mycoplasma genitalium* (Baseman et al. 1995), and this may explain the high prevalence and persistence of *M. bovis* inside host cells. Using a monoclonal antibody (MAb), the existence of *M. bovis* inside macrophages, neutrophils and necrotic cell debris in alveolar walls has been demonstrated (Rodriguez et al. 1996). They suggested that this may explain the isolation of the organism from blood and its systemic dissemination and subsequent joint colonisation.

Many herds may be infected with *M. bovis* and infections can range from subclinical to severe clinical disease. Clinical mastitis may result in production losses (Brown et al. 1990), decreased milk quality, dysfunctional quarters (Mackie et al. 1986), and further problems associated with the treatment and control of infections in a herd. Infections with *M. bovis* are highly resistant to therapy. Indeed, the presence of clinical mastitis that is resistant to antibiotic therapy is generally regarded as one indicator that the causal organism is *Mycoplasma* (Kirk and Lauerman 1994). In the light of such treatment failure, the present

method for controlling *Mycoplasma* infection in herds is usually the culling of all animals detected to be shedding the organism. Detection of "shedders" at an early stage, by using single animal diagnosis, is considered essential if control is to be effective (Sachse et al. 1993).

The diagnosis of *Mycoplasma* mastitis on clinical symptoms alone is difficult as the disease often develops insidiously and will advance quickly to become chronic in nature (Stalheim 1983). The current methods for the detection of *M. bovis* are restricted to culture and serology. Both methods are labour intensive and complex, and poor sensitivity is commonly experienced (Simecka et al. 1992). Isolation and identification may take weeks, and few laboratories have the expert skills required to culture *Mycoplasma* routinely. Serodiagnosis requires the demonstration of increasing antibody titre that may peak at ten to fourteen days after the onset of clinical symptoms. Consequently, the pathogen cannot be detected during this incubation period. Moreover, an additional problem with detection by serology is the cross reactions among *Mycoplasma* species.

The absence of practical and rapid detection methods, and resistance of the organism to conventional antibacterial therapy has previously prevented the determination of the extent of *M. bovis* mastitis and this has hampered effective control of the disease. The development of a sensitive and specific assay for the detection of *M. bovis* is required. Assays for detection of *M. bovis* based on DNA hybridisation and polymerase chain reaction (PCR) have been developed, but they have lacked either specificity (McCully and Brock 1992), or sensitivity (Hotzel et al. 1993).

The present study was carried out with the following aims:

- to develop a robust, sensitive, specific and economical serological assay for the detection of *M. bovis* infection in cattle;
- to develop methods for the isolation and characterisation of *M. bovis*;
- to develop rapid, sensitive and specific diagnostic hybridisation and PCR methods for the detection of *M. bovis* in biological samples;
- to apply and evaluate these methods in the field by undertaking a pilot study to investigate the prevalence of *M. bovis* infection in cattle in north Queensland.

Completing these aims would provide a rapid and specific detection of *M. bovis* at acceptable costs, a better understanding of the extent of *M. bovis* infection and its association with disease, and an ability to control the spread of *M. bovis* mastitis by its early recognition.

CHAPTER 2
LITERATURE REVIEW

2.1 History of *Mycoplasma*

Mycoplasma have great historical significance in the history of cattle disease since contagious bovine pleuropneumonia (CBPP) was eventually shown to be caused by a *Mycoplasma* spp. It was this disease that played an important role in the stimulation of research into the control and eradication of infectious diseases in animals (Stalheim 1983).

Contagious bovine pleuropneumonia was first described by Louis Pasteur in Europe and, in 1898, Nocard and Roux cultivated the disease agent in the peritoneal cavity of rabbits. In 1900, Dujardin-Beametz successfully cultured the agent of the disease on a solid microbiological medium supplemented with serum as described in Masover and Hayflick (1981). This organism is now called *Mycoplasma mycoides* subsp. *mycoides* and is the type species of the genus *Mycoplasma*. Initially, *Mycoplasma* were considered viral, as they could pass through filters that removed other bacteria from solution. With elucidation of the true nature of viruses in the 1930s, it became clear that *Mycoplasma* could not be defined as a true virus (Razin 1992). A major stimulus of interest in this field occurred in 1935 when Klieneberger-Nobel cultured *Streptobacillus moniliformis* and suggested the L-phase variants of bacteria were *Mycoplasma,* that lived in symbiosis with bacteria. L-forms are mutants of bacteria that have partially or entirely lost their cell wall. They can also be induced by laboratory manipulations of bacterial cultures in the presence of antibacterials that inhibit bacterial cell wall growth (Masover and Hayflick 1981).

This similarity led to much speculation that *Mycoplasma* were stabilised L-forms of bacteria. It took years to correct this misconception, following investigations into the structure, composition and biosynthesis of bacterial cell walls. The recent advances in molecular biology have provided G+C content and DNA-DNA hybridisation data that have excluded any relationship between *Mycoplasma* and the stable forms of distinguished bacteria with cell walls (Razin 1969).

2.2 Classification

2.2.1 The *Mycoplasma* genome

Mycoplasmas are small, from 0.1 to 0.3 μm in diameter and up to 98 μm in length, and the simplest self-replicating organisms. They have no cell wall (Razin 1987). Their small size explains their characteristic ability to pass through a 450-nm (and often a 220-nm) membrane filter.

The basis for the present *Mycoplasma* classification system was suggested by Edward and Freundt (1956). Families and genera are distinguished primarily by means of the following properties: genomic size, requirement for cholesterol, shape, NADH oxidase location, the urease reaction, habitat, absence of reversion to a bacterium, and the effect of oxygen and temperature. *Mycoplasma* is now the communal name for the Class *Mollicutes*, a name derived from the Latin mollis (soft) and cutis (skin) to describe the lack of a rigid cell wall in these organisms. This class consists of more than 120 species from eight different genera: *Mycoplasma*, *Ureaplasma, Entomaplasma, Mesoplasma, Spiroplasma, Acholeplasma, Anaeroplasma* and *Asteroleplasma* (Tully et al. 1993). Bovine pathogens are within the genera *Mycoplasma* and *Ureaplasma.* These two genera belong to the same Family *Mycoplasmataceae* within the Order *Mycoplasmatales* (Table 2.1).

The mole percent G+C of prokaryotic DNA is an effective taxonomic tool. A difference in the G+C content greater than 1.5 to 2.0 mol% in the DNA of two bacteria is sufficient to distinguish them as different species (Jounson 1984). *Mycoplasma* contain a single circular chromosome of double stranded DNA that has a G+C content of 23 to 41 mol%. DNA renaturation studies indicate a genome size of only 750 kb or almost one sixth the size of the *E. coli* chromosome (Razin and Freundt 1984).

Based on comparative analysis of 16 S rRNA sequences derived from almost 50 species, it is believed that *Mycoplasma* are monophyletic and arose from the *Lactobacillus* branch (Woese et al. 1980). They lost their cell wall during evolution from Gram-positive walled bacteria, together with a significant reduction in genome size. Moreover they may have lost many metabolic activities including the ability to synthesise nucleic acid precursors (Masover and Hayflick 1981; Maniloff et al. 1982).

Table 2.1 Taxonomy of the Class *Mollicutes*.

Classification	Named species	Genome size (Kbp)	Mol% G + C of DNA	Cholesterol requirement	Habitat
Order I: *Mycoplasmatales*					
Family I: *Mycoplasmataceae*					
Genus I: *Mycoplasma*	100	580 – 1300	23 – 41	+	Humans, animals, plants, insects
Genus II: *Ureaplasma*	6	730 – 1160	27 – 30	+	Humans, animals
Order II: *Entomoplasmatales*					
Family I: *Entomoplasmataceae*					
Genus I: *Entomoplasma*	5	790 – 1140	27 – 29	+	Insects, plants
Genus II: *Mesoplasma*	12	870 – 1100	27 – 30	–	Insects, plants
Family II: *Spiroplasmataceae*					
Genus I: *Spiroplasma*	17	1350 – 1700	25 – 31	+	Plants, insects
Order III: *Acholeplasmatales*					
Family: *Acholeplasmataceae*					
Genus I: *Acholeplasma*	13	1600	27 – 36	–	Animals, plants, insects
Order IV: *Anaeroplasmatales*					
Family: *Anaeroplasmataceae*					
Genus I: *Anaeroplasma*	4	1600	29 – 33	+	Rumen of cattle and sheep
Genus II: *Asterolplamsa*	1	1600	40	–	Rumen of cattle and sheep

Adapted and modified from Whitcomb et al. (1995).

2.3 Morphology

2.3.1 Cell envelope

While *Mycoplasma* possess most of the biological characteristics of bacteria, they do not have a cell wall as they cannot synthesise the peptidoglycan layer associated with bacterial cell walls. A phospholipid layer with associated protein, which is similar to the cytoplasmic membrane of other bacteria, surrounds the cytoplasm of *Mycoplasma*, and this may be surrounded by a capsule or capsule like structures (Razin 1992). The cell membrane of mycoplasmas appears to contain substantial amounts of polysaccharide composed of hexoses, often including hexosamines (particularly glucosamine and galactosamine) and N-acetyl glucosamine. This layer has been termed a capsule, but its detachment from the membrane is difficult, suggesting that it may be hydrated and probably plays a structural role, compensating in particular for the lack of a peptidoglycan wall (Rosenbusch and Minion 1992). The capsule can be seen with the aid of a polycationic compound such as ruthenium red. Some *Mycoplasma* have attachment organelles, such as a nap and a cytoskeleton that may play a role in motility and attachment of organisms to substrate (Taylor-Robinson and Bredt 1983).

For many years the collective term "pleuropneumonia-like organism" (PPLO) was used for *Mycoplasma*. The term *Mycoplasma*, suggested by Nowvak (1929), was based on the morphology of *Mycoplasma mycoides,* the agent of CBPP, that produces long branching filaments. Because of the filaments' similarity to fungi, the names "myco" which means fungus, and "plasma" which refers to something formed or moulded, was used. *Mycoplasma* cells vary in size within a single culture from those that are easily visible by ordinary light microscope, to those too small to be seen. The morphologic appearance is variable, depending upon the species, the growth phase, preparation and/or the growth medium. They have high concentrations of cholesterol or other sterols in the cell envelope which allow them to be very stable and flexible and thus vary in shape from cocci and spirals to filaments and rings (Masover and Hayflick 1981; Boyd 1984). These properties, moreover, permit cells to be easily deformed so that they can squeeze through the mucous layer of host cells (Quinn et al. 1994).

2.3.2 Mycoplasma as an extracellular and intracellular parasite

Mycoplasma are among the most nutritionally fastidious microorganisms because of the limited size of their genome and the reduced number of enzyme systems they possess. They are perfect parasites that infrequently kill their host (Razin 1994), and can retain both extracellular and intracellular biological functions (Taylor-Robinson et al. 1991). There is considerable variability between species in their ability to penetrate the mucous layer, intracellular spaces or membrane crypts of epithelial cells. They can enter host cells, establish residence, and persist *in vivo*. It has been shown that *Mycoplasma* are generally localised in the cytoplasm around the nucleus and remain viable in this location (Baseman et al. 1995)

2.3.3 Growth requirements and culture

Mycoplasma are not readily propagated outside animals cells. For *in vitro* culture they require enriched media containing cholesterol from serum supplement, and nucleic acid precursors from yeast extract. Because of common antigens and subsequent cross reactions between *Mycoplasma* species, the serum must be from *Mycoplasma*-free animals (Jasper 1981; Stalheim 1984), otherwise the growth of *Mycoplasma* is inhibited by heterologous antibody (Kirchhoff et al. 1982). Growth rates vary for the different *Mycoplasma* species and only slight turbidity is produced in broth media. The fastidious growth requirements of these organisms explains the observed common failure to detect them, especially when they are present in low numbers. The presence of bacteria or other *Mycoplasma* species, e.g. *M. bovirhinis*, which grow faster and produce acidification of media, may inhibit the isolation of *M. bovis*. Hence the addition to the media of immune serum against strains that grow rapidly may be necessary (Gourlay and Howard 1979). Moreover, the presence of specific antibodies and *Mycoplasma* inhibitors, such as lysolecithin, that may be present in the specimens (joint fluids, semen, and tissues) may also prevent the growth of *Mycoplasma* (Frey 1986; Ter-Laak et al. 1992a). Dilution of samples is sometimes necessary and both undiluted specimens and tenfold dilution in *Mycoplasma* broth (MB) (up to 10^{-6}) should be cultured (Gourlay 1981). It has been the frustrating experience of many workers to know that *Mycoplasma* organisms must be present in a specimen but to be unable to culture them due to their specific growth requirements.

2.3.4 Growth on solid media

Growth on solid media requires a moist atmosphere and a temperature range of 36° – 38°C with 5-10% CO_2 enrichment. Colonies formed on agar vary in size from microscopic to those that can be seen without magnification (0.1-0.6 mm). The range in colour is from clear or almost transparent, to brown. When viewed with moderate magnification (x 20), they typically have a nipple or fried-egg appearance. The dense centre is caused by the organisms growing down into the agar and represents a greater accumulation of cells. This appearance is due to their small size and lack of a rigid cell wall, which allows *Mycoplasma* to pierce and grow in the interstices of the agar fibrils forming the solid media (Masover and Hayflick 1981). Other types of colony forms are not unusual and may include smooth convex, granular or lacy flat colonies. Colonial forms may change with different culture media and growth conditions, and between original and cloned isolates (Madden et al. 1967).

2.4 Ecology of *Mycoplasma*

Mycoplasma are essentially host-specific and may cause disease in a wide range of hosts, for example, *M. bovis* has been isolated from the pneumonic lungs of goats and human (Stalheim 1984). Many different species of *Mycoplasma* have now been isolated from different animals including aquatic animals, man and also from insects and plants (Kirchhoff et al. 1987; Razin 1992). A number of species have been found to cause serious disease in their host, others are associated with disease conditions but their potential to cause disease is unknown. These diseases extend from subclinical disease that affects only reproduction, to severe disease that results in pain and death of the animals (Simecka et al. 1992). This may be of major economic importance because of high morbidity and mortality rates and reduced production from cattle, sheep, goat, swine and poultry (Stalheim 1984).

2.4.1 *Mycoplasma* in domestic animals

There is convincing information that various *Mycoplasma* species can produce several diseases in cattle. Infections in cattle with which *Mycoplasma* are associated can be divided into five groups on an anatomic basis. These are respiratory infection, mastitis, arthritis, urogenital infection, and eye infection (Gourlay 1973). Because a number of different *Mycoplasma* species have been isolated from each of these sites during infection, the

infections themselves can be further subdivided based on *Mycoplasma* species. The result of numerous studies to fulfill Koch's postulates indicate an aetiologic role for *M. mycoides*, *M. bovis* and *M. dispar* (Simecka et al. 1992). This review will concentrate on the diseases produced by the most prevalent *Mycoplasma* in cattle.

2.4.2 *Mycoplasma mycoides*

Mycoplasma mycoides subspecies *mycoides* is the cause of a devastating chronic disease in cattle known as contagious bovine pleuropneumonia. It was once widespread and the cause of heavy losses in many areas of the world. This disease is known to be present now only in Africa, France, Spain, Portugal, Italy, the Iberian Peninsula and India (Ross and Young 1993; Nicholas and Bashiruddin 1995) and has been eradicated from other areas of the world. It was introduced to Australia in 1858 and spread through six states, before being finally eradicated. The last clinical case occurred in 1967 and freedom from the disease in Australia was declared in 1973 (Hungerford 1990).

The disease is characterised by bronchopneumonia with gross serofibrinous pleurisy. Morbidity can be as high as 100% in a susceptible herd and mortality varies from 10 to 90% (Cottew and Leach 1969). About 10 to 30% of cattle are apparently completely resistant to natural or experimental infection. About 50% become clinically infected, 20% develop subclinical infection, and 10% may become chronic carriers. It is believed that the subclinical and chronic carrier states are the most dangerous form for the prevention and control of disease because they increase its spread (Gourlay 1973).

2.4.3 *Mycoplasma bovis*

With the exclusion of *M. mycoides, M. bovis* is considered the most pathogenic and economically important *Mycoplasma* species of cattle in the world (Gourlay and Howard 1979; Thomas et al. 1986). This organism was originally recovered in pure culture from the mammary gland of a cow affected with severe mastitis (Hale et al. 1962). It was called *M. agalactiae* var. *bovis* because of its association with a typical clinical syndrome of acute infectious mastitis that spreads rapidly through a herd and results in dramatic loss of milk. The organism was classified subsequently as a separate species, *Mycoplasma bovis*, based on phenotypic characterisation and nucleic acid hybridisation studies (Askaa and Erno 1976).

The pathogenicity of *M. bovis* has been shown for a variety of anatomical sites under controlled experimental condition. The available evidence suggests that *M. bovis* alone can cause mastitis (Jasper et al. 1987), arthritis (Simecka et al. 1992), pneumonia (Thomas et al. 1986) and genital disease in both bulls (LaFaunce and McEntee 1982) and cows (Bocklisch et al. 1986).

2.5 *Mycoplasma* Mastitis

The bovine mammary gland may be more vulnerable to *Mycoplasma* infection than any other site, although *Mycoplasma* is not normal resident microflora and it has not normally any resident microflora. Thus, any mastitis from which pathogenic *Mycoplasma* are isolated in large numbers can probably be safely presumed to be caused by that *Mycoplasma* (Gourlay and Howard 1979). During the past few years, serious outbreaks of mastitis caused by *Mycoplasma* have been reported in cattle in many areas of the world. The first reported case of mastitis associated with *Mycoplasma* was a rare form caused by *Mycoplasma bovigenitalium* (Stuart et al. 1960). However, at least 12 species of *Mycoplasma* have been isolated from the milk of dairy cattle (Table 2.2). The most common species causing clinical mastitis is *M. bovis* (Jasper 1982; Bushnell 1984).

2.5.1 Prevalence

Mycoplasma-induced mastitis is widely distributed (Kirk and Lauerman 1994). The disease seems different from animal to animal in terms of the severity, number of quarters involved, and duration of the infection. Many herds are infected with this organism, and symptoms range from subclinical to severe clinical disease (Simecka et al. 1992; Kirk and Lauerman 1994). Severe cases of mastitis are less common than are mild or subclinical infections (Jasper 1982).

Table 2.2 *Mycoplasma* species associated with mastitis.

Organisms	Naturally occurring mastitis	Prevalence	Experimentally induced mastitis	Source
M. bovis	Moderate to severe	High	Severe to moderate	Hale et al. (1962)
M. bovigenitalium	Moderate to severe	Rare	Moderate	Stuart et al. (1960)
M. bovirhinis	Moderate	Rare	Moderate	Jasper (1981)
M. arginini	Moderate nonclinical	Rare	Moderate	Jasper (1981)
M. californicum	Severe	Rare	–	Dellinger et al. (1977)
M. dispar	–	–	Moderate	Brownlie et al. (1976)
M. canadence	Moderate	Rare	–	Jackson (1981)
M. alkalense	Moderate	Rare	–	Jasper (1981)
M. capricolum	Severe	Rare	–	Taoudi and Kirchhoff (1986)
M. group 7	Severe	Rare	Severe	Alexander et al. (1985)
Ureaplasma	–	Rare	Moderate	Jasper (1981)
M. F38	–	Rare	–	Kumar and Gard (1991)

2.5.2 Clinical features

All clinical signs of *Mycoplasma* mastitis are similar, but mastitis caused by *M. bovis* is usually more severe than other *Mycoplasma* species. The dynamics of mastitis within the herd are illustrated in Figure 2.1. Both subclinical and clinical mastitis can occur and the severity of the clinical symptoms of *M. bovis* infection depends on the age of the animal (younger are more susceptible than older animals), and previous exposure to *M. bovis* infection. Some degree of immunity may prevent the appearance of severe clinical symptoms caused by *M. bovis* (Bennett and Jasper 1978a; Jasper 1982). Subclinical infections are those for which no visible changes occur in the milk or the udder, but milk production may decrease, bacteria are present in the secretion, and the composition of milk is changed. However, occasionally cows may shed *Mycoplasma* in the milk without any change in milk production (Jasper 1981; Brown et al. 1990).

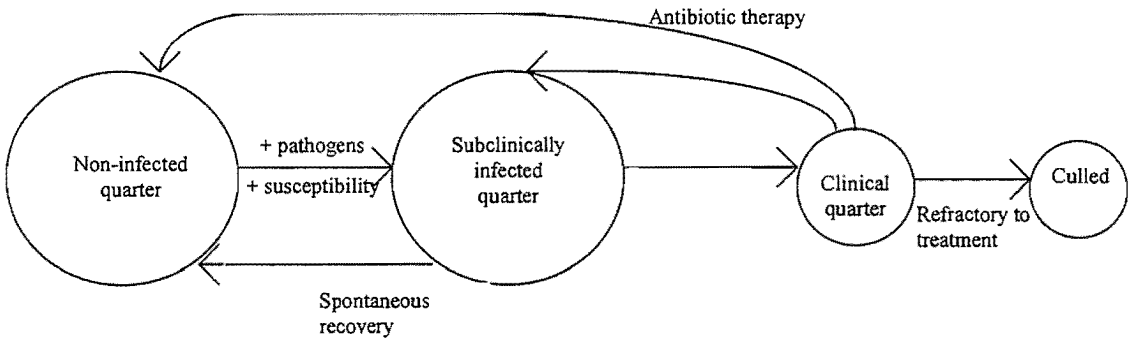

Figure 2.1 Pattern of clinical and subclinical mastitis in a dairy herd [modified from Bramley (1992)].

Typical clinical *Mycoplasma* mastitis is characterised by a marked decrease in milk production, abnormal milk (which is often yellow or brown to tan with a flaky deposit in a watery or serous fluid) and is resistant to therapy (Jasper 1981). Often two quarters on the same side become affected. Systemic signs, such as elevated rectal temperature, lethargy or anorexia are usually transient and may go undetected. On the whole, the severity of clinical signs is different (Ter-Laak et al. 1992b) depending on sanitation and the influence of secondary bacterial infection during the *Mycoplasma* infection. The disease may be more

severe during early rather than late lactation. The somatic cell count (SCC) is elevated in individual cows infected with subclinical mastitis. The increase in somatic cells usually occurs within a few days of the onset of infection. Nevertheless, *M. bovis* can be isolated from the mammary gland and milk with an increased or normal cell count (Wentink et al. 1987). Most of the increase in SCC is due to neutrophils (Jasper 1981). Antibiotic therapy often hastens abatement of symptoms and cows appear to recover after several days or weeks but they continue to shed *Mycoplasma* in their milk for a variable period, and perhaps intermittently (Jasper 1977). This variable duration of clinical signs and shedder status contributes to difficulty in predicting the outcome of treatment of infected quarters or the determination of full bacteriological recovery. For this reason cows diagnosed positively for *M. bovis* mastitis should be considered as potentially positive for life (Jasper 1981).

2.5.3 Route of infection

Herd outbreaks are often traced back to the introduction of infected cows to a herd (Jasper 1981; Gonzalez et al. 1993). Heifers that have never been milked may be recognised to be infected during their first milking and maybe the source of *Mycoplasma* entry into the herd (Jasper 1981). Cows at any stage of lactation, including dry cows, can be affected, but most infections occur during the milking process. Although the usual route of infection for cows is through the streak canal, as in typical bacterial infection (Jasper et al. 1987), there is evidence that suggests that the disease can be spread haematogenously. Recovery of *M. bovis* from many tissues through the body including the lung, vagina, foetus, liver, kidney, blood, spleen, joints, lymph nodes and elsewhere, suggest that systemic invasion occurs (Jain et al. 1969; Stalheim and Proctor 1976; Pfutzner and Schimmel 1985; Ross 1985). Further evidence for haematogenous spread came from one carefully controlled study aimed at preventing external spread from quarter to quarter during milking. *Mycoplasma bovis* mastitis developed in the non-inoculated quarters of four cows that were all inoculated in a single quarter and mycoplasmaemia was also demonstrated in three of the four cows in this study (Bennett and Jasper 1978a). Also, the isolation of *Mycoplasma* from the cows without a history of infection also suggests the systemic spread of the disease (Bennett et al. 1977). It has been suggested that the respiratory tract may play an important role in the spread and invasion of this organism, as *M. bovis* has been isolated from the blood of calves within a week following tracheal inoculation (Thomas et al. 1986). Calves also may become infected by drinking contaminated milk (Stalheim and Page 1975; Alexander et al. 1985).

2.5.4 Pathophysiology of the bovine mammary gland

An inflammatory response is initiated when bacteria enter the mammary gland and multiply in the milk (Eberhart et al. 1987; Jasper et al. 1987). Bacterial toxins, enzymes, cell membranes and cell-wall components may affect the function of the mammary epithelium but also excite the production of numerous mediators of inflammation by inflammatory cells that may be directly involved in the pathogenesis of the disease (Gallin et al. 1992). These mediators, including complement components (Kehrli et al. 1991; Gallin et al. 1992), prostaglandins (Giri et al. 1984; Anderson et al. 1985), leukotrienes (Rose et al. 1989), histamine (Zia et al. 1987), serotonin, interleukins (Daley et al. 1991; Shuster et al. 1993), tumour necrosis factors (Babiuk et al. 1991; Shuster et al. 1993), interferon (Babiuk et al. 1991) and other cytokines (Babiuk et al. 1991; Daley et al. 1991; Kehrli et al. 1991), lead to inflammation that increases vascular permeability, vasodilation, oedema, increased blood flow, neutrophil margination and leakage of blood components into milk (McFadden et al. 1988; Shuster et al. 1991). This also involves the movement of some normal milk components out of the alveolar lumen into the perivascular space, decreased mammary synthetic activity, pain, and fever (Gallin et al. 1992).

The accumulation of leukocytes causes the formation of clots, and blood clotting factors may block small ducts and hinder complete milk removal (Eberhart et al. 1987). Injury to epithelial cells and blockage of small ducts can result in the creation of scar tissue occasionally, with the subsequent permanent loss of function of that part of the gland. In other cases, inflammation may decline, tissue repair may occur, and cell function may return in that or a subsequent lactation.

2.5.5 Inflammatory responses of the mammary gland

The inflammatory responses of the mammary gland to infection are primarily initiated by the influx of polymorphonuclear neutrophil (PMN) leukocytes into the mammary gland (Jasper 1981). Polymorphonuclear leukocytes normally flow freely within capillaries with only minimal adherence to vessel walls. During infection and inflammation, adhesion molecules are expressed, and PMN leukocytes adhere to the endothelium of the smaller blood vessels and pass between cells lining the vessel. Chemical messengers or chemotactic agents released

from leukocytes normally in the milk or from damaged secretory cells attract PMN into the milk in large numbers (Craven and Williams 1985; Nickerson 1985).

The PMN appear in large numbers, lining the outside of some alveoli (Nickerson and Pankey 1984; Harmon and Langlois 1989). In other areas, damage to milk-synthesising cells may be obvious, and masses of PMN may pass between epithelial cells into the lumen of the alveolus. Thus, the result of these processes is an increase in the SCC in milk resulting from PMN migration to the site of infection. The major function of PMN in milk is to capture and digest the invading bacteria (Nickerson and Pankey 1984; Craven and Williams 1985).

The leukocytes in milk may also release specific substances that change the permeability of blood vessels or attract more leukocytes to the area to combat the infection (Craven and Williams 1985; Gallin et al. 1992). With persistent bacterial infections, leukocyte numbers may fluctuate, but usually remain abnormally high. Such abnormal numbers of somatic cells continue after bacteria are eliminated or until healing of the gland occurs. However, either experimental or natural *M. bovis* infections may or may not be accompanied by inflammation (Bennett and Jasper 1978a; Jasper 1981).

2.5.6 Somatic cell count in milk

Milk somatic cells are primarily leukocytes, which include macrophages, lymphocytes, and PMN. The main function of these cells is to protect against infectious diseases. Studies identifying cell types in milk have shown that epithelial cells rarely occur in udder secretions (including those from the dry gland), and range from zero to 7% of the cell population (Lee et al. 1980). Therefore, increases in SCC at the end of lactation are not from increased sloughing of epithelial cells. The major factor affecting SCC and decreased milk production at the quarter, cow, or bulk tank level is an infection of the mammary gland (Dohoo and Meek 1982; Eberhart et al. 1982).

2.5.6.1 Quarter level

Somatic cell counts from normal quarters are almost always below 200 x 10^3/mL, but may be below 100 x 10^3/mL during first lactation. One study estimated that 50% of uninfected cows have SCC under 100 x 10^3/mL, and 80% have under 200 x 10^3/mL (Eberhart et al. 1979). An

elevated count is abnormal and an indication of inflammation in the udder (Sheldrake et al. 1983).

Most studies indicate that the use of SCC alone to categorise quarters as infected or uninfected will give both false positive and false negative results (Dohoo and Meek 1982). These errors may be in part due to the normal fluctuation of SCC observed during an infection. Temporary changes in SCC following experimental challenge with bacteria of mammary glands (Erskine et al. 1989; Sears et al. 1990; Shuster et al. 1991, 1993) suggest dramatic changes in the early acute stages of the infection, reaching a peak SCC within hours or days, depending on the kind of infection. The peak may be followed by a modest reduction in SCC as bacteria are killed by PMN. The decline in SCC can vary considerably and may be dependent upon the bacteriological outcome of the infection, the pathogen involved and differences between individual cows. With chronic infections, SCC and bacterial number tend to fluctuate with time (Sears et al. 1990). The measure of SCC responses to infections differs among cows, and discrimination between the types of pathogens is not possible using SCC alone (Dohoo and Meek 1982).

2.5.6.2 Composite milk

The SCC in a composite milk sample is related to the number of quarters infected and milk being produced by each quarter. However, if none of the quarters are infected, generally an SCC below 250 x 10^3/mL would be considered normal for composite milk samples.

2.5.6.3 Bulk milk

Bulk milk SCC (BMSCC) in a herd of cows with physiologically normal quarters should be below 200 x 10^3 cells/mL (Table 2.3), but evidence shows that even in these herds there may be some subclinical infection (O'Rourke and Blowey 1992). A BMSCC of 250 x 10^3 cells/mL is considered a practical upper limit for herds with mastitis under control (Table 2.4).

Table 2.3 Ranges of somatic cell counts related to numbers of infected quarters and cows [Pearson and Greer (1974)].

SCC (x 10^3 cells/mL)	No. of herds	Infection in quarters (%)	Infection in cows (%)
219 – 490	12	9.61	25.8
535 – 789	12	17.76	42
1005 – 1700	5	29.54	54.4

Table 2.4 Guidelines for interpretation of bulk milk cell counts and the estimated mastitis level present [O'Rourke and Blowey (1992)].

Bulk milk somatic cell count x 10^3/mL	Estimate of mastitis problem
<250	Slight
250 – 490	Average
500 – 750	Above average
750 – 1000	Bad
>1000	Severe

The effects of other factors that may affect SCC, including stage of lactation and age (Eberhart et al. 1979), season (Hogan et al. 1989) and various stresses (Paape et al. 1973; Wegner et al. 1976), are usually minor if the gland is uninfected.

2.5.6.4 Somatic cell count and M. bovis infection

During *M. bovis* infection, the major increase in SCC is due to an influx of PMN into the milk. The quarters infected with *M. bovis* may have a variable reaction, with the degree and the nature of the cellular response likely to be proportionate to the severity of infection (Boothby et al. 1986a). Most reports indicate that the dominant cells (90%) are neutrophils but eosinophils in the alveoli have also been described (Geary et al. 1981; Jasper 1982). Somatic cell counts from 86 x 10^4 to 86 x 10^6/mL in milk from cows with naturally occurring *M. bovis* infections have been reported (Jasper et al. 1966; Jasper 1981).

It has been speculated that the large influx of neutrophils into the udder may result in a marked leucopaenia of the peripheral blood within 48 hours. A moderate leucopaenia,

sometimes accompanied by a degenerative left shift and toxic neutrophils, has been described. This was shown to persist for 5-12 days, after which a moderate leukocytosis and neutrophilia were present (Jain et al. 1969). A neutropaenia that lasted up to two weeks has been reported in cows experimentally infected with *M. bovis* (Jasper 1981).

2.6 *Mycoplasma* Respiratory Infections in Cattle

2.6.1 Introduction

Following investigation into the aetiology of respiratory disease in cattle, Carter (1954) isolated *Mycoplasma* from the lungs of cattle for the first time. Since then many different *Mycoplasma* species have been isolated from the respiratory tract of cattle (Ross 1985). These species are *M. bovis* (Stalheim 1983; Gourlay and Houghton 1985; Ter-Laak et al. 1992a), *M. bovirhinis* (Muenster et al. 1979; Ter-Laak et al. 1992a), *M. dispar* (Knudston et al. 1980), *M. alkalescens* (Gourlay and Howard 1979), *M. bovigenitalium* (Cottew 1970; Ter-Laak et al. 1992a), *M. arginini* (Muenster et al. 1979), *A. laidlawii* (Gourlay and Howard 1979), *A. modicum* (Gourlay and Howard 1979; Tinant et al. 1979), and *Ureaplasma* (Knudston et al. 1980). The results of many experimental studies in pathogen-free animals indicate an aetiologic role for *M. bovis*, *M. dispar* and *Ureaplasma* species. They have been shown to be pathogenic in the absence of viruses or other agents, as shown in experimental infections of gnotobiotic calves (Friis 1980; Houghton and Gourlay 1983; Howard 1983; Gourlay and Houghton 1985; Thomas et al. 1986). Among these three *Mycoplasma*, *M. bovis* is the most common species associated with the bovine respiratory tract worldwide. It causes the most severe lesions and has the highest mortality, but disease outbreaks are sporadic (Thomas et al. 1986; Brys et al. 1989; Ross and Young 1993; Rodriguez et al. 1996).

2.6.2 Routes of infection

There are two routes by which pathogenic organisms may enter the lungs: bronchogenicly, which is the most usual, and haematogenously (Brys et al. 1989). By the bronchogenic route, the disease may appear during birth when *Mycoplasma* colonise most calves, first in the nose, vagina and upper respiratory tract, and later in the lower respiratory tract. At necropsy, lung lesions are usually limited to a few lobules in apical or cardiac lobes with consolidation of a small numbers of lobules. The lesions can be sharply distinguished from adjacent ones (Jones

et al. 1989). It is believed that the respiratory tract plays an important role in the spread of *Mycoplasma* infection to the target organs. Many infections become chronic or latent in the lungs, and therefore facilitate later spread to new susceptible animals (Gourlay and Howard 1978; Ross 1985).

2.6.3 Pathophysiology of the upper respiratory tract in cattle

The respiratory tract is constantly exposed to air that contains infectious materials. Inhaled particulate matter that ranges in diameter from two to 10 microns is deposited in the airways from the larynx to the terminal bronchioles lined by mucus-covered ciliated epithelium. This inhaled material is removed by the mucociliary transport system or by the cough reflex mechanism. The ciliated airways transport this mucus-particulate matter to the oropharynx, to be swallowed there, at a rate of approximately 15 mm/min (Dulfano 1973). Ciliated cells extend down to the terminal bronchioles, but their numbers are few in contrast with those present in the major bronchi and trachea. Intercellular connections between ciliated cells allow coordination of movement, primarily upwards to the oropharynx. Disruption of the action of ciliated cells by infectious agents, high levels of gaseous irritants, or extremes in temperature or humidity may hinder flow rate (Vestweber 1986).

The important physical properties of mucus are viscosity and elasticity. Infected mucus resulting from bronchitis is moved abnormally because of increased viscosity and reduced elasticity. Mucus is made up of two layers: an outer gel layer derived originally from goblet cells, and an inner sol layer secreted by submucosal glands (Kilburn 1967). Mucus serves to prevent epithelial dehydration and as a protective barrier against some infectious agents (Negus 1963). Ninety percent of the material deposited on the mucociliary blanket is cleared effectively (within one hour) from the respiratory tract. Several factors, including dehydration, cold weather, irritant gases (such as ammonia), abnormal body electrolytes (metabolic acidosis), anaesthetics, general hypoxaemia and infectious agents, will impede mucociliary activity (Dulfano 1973; Thomson and Gilka 1974). If the presence of these factors allows pathogenic bacterial agents to colonise or remain in the upper respiratory tract for three to four hours, infection probably will occur.

2.7 *Mycoplasma* Arthritis in Cattle

The joints are a major site of localisation and damage for a majority of *Mycoplasma* species (Adler 1965). Joint localisation by *Mycoplasma* is a form of septicaemia and suggests the highly invasive nature of *Mycoplasma* (Jasper 1967; Gourlay 1973). So far several *Mycoplasma* species including *M. bovis* (Wentink et al. 1987; Simecka et al. 1992), *M. group* 7 (Shiel et al. 1982; Whithear 1983; Alexander et al. 1985), *M. alkalescens* (Gourlay et al. 1976; Bennett and Jasper 1978b), and *M. mycoides* subsp. *mycoides* (Harbi and Salih 1979) have been isolated from arthritic cattle. Nevertheless, *M. bovis* is the most commonly recognised arthritogenic mycoplasmid agent in cattle (Wentink et al. 1987; Simecka et al. 1992).

2.7.1 Route of infection

Mycoplasma probably enter the blood stream from the lungs and localise in synovial membranes (Radostits et al. 1988). Oral infection via the milk from an animal with mastitis may also occur. Outbreaks of disease usually occur in feedlots and are often preceded by management stress (Jubb et al. 1993).

2.7.2 Clinical features

There is variability in the severity and consequences of arthritis due to *M. bovis*. Some animals develop only a mild condition with no fibroid response, while others develop chronic arthritis with a tendency to recur. Mildly affected cattle recover, but more severely affected cattle with signs of joint swelling that are warm to the touch do not appear to respond to antibiotic treatment (Radostits et al. 1988), become chronically lame (Wentink et al. 1987), and may finally have to be culled. The disease is characterised by tenovaginitis, pretendonitis and synovitis with multiple joint effects. In natural infections one or two joints in one limb may be affected, or more than one limb may be involved although usually severe lesions develop in one limb only. In acute disease, the joints are swollen by opaque cream-coloured fluid that contains flecks of fibrin and synovia are red and swollen (Jubb et al. 1993).

Experimental infection with *M. bovis* can result in destruction of cartilage and development of severe arthritis in the cervical spine (Royan et al. 1983; Cole et al. 1985a). Most animals

recover in one to two months, but a few develop chronic arthritis and must be culled. Isolation of *Mycoplasma* is relatively easy in the acute disease but after a week or two the high concentration of specific antibodies in the synovia may hamper the isolation of the organism (Frey 1986). Arthritic calves may have a fever, joint swelling, lameness, and a neutrophilia (Royan et al. 1983). In progressively worsening fibrinous synovitis, arthritis involves generally the large joints such as the stifle, shoulder, hock, carpus and elbow (Radostits et al. 1988).

2.8 Reproductive Disease

Reproductive productivity in a cattle enterprise is a function of good management. Many *Mycoplasma* species have been isolated from various parts of the genital tract of bulls and cows with reproductive problems (Edward et al. 1947; Hoare and Haig 1964; Gourlay and Howard 1979; Saed and Al-Aubaidi 1982). *Mycoplasma bovis* is often associated causally with genital tract disease. It has been shown experimentally to cause purulent endometritis, (Kreusel et al. 1989) salpingitis and salpingoperitonitis in pregnant heifers, localised peritonitis (Hartman et al. 1964; Kreusel et al. 1989), sterility or low fertility, ovarian adhesions and abortions (Hirth et al. 1966; Stalheim and Proctor 1976; Bocklisch et al. 1981; Bocklisch et al. 1986). In bulls it can cause seminal vesiculitis, epididymitis, and orchitis (Doig 1981; LaFaunce and McEntee 1982) and it is repeatedly isolated from semen (Bell 1984; Kirkbride 1987), where it causes decreased fertility of spermatozoa (Eaglesome and Garcia 1990). In addition, *M. bovis* can survive in antibiotic treated frozen semen (Hirth et al. 1967). Although the incidence of infection of semen samples has been reported as low (Eaglesome and Garcia 1990), in another study *M. bovis* was isolated from more than one third of semen samples from 181 bulls (Kirkbride 1987).

2.9 Virulence Mechanisms of *Mycoplasma bovis*

2.9.1 Introduction

Mycoplasma bovis possesses several characteristics that enable it to be a pathogen. The mechanisms by which these organisms cause damage are thought to be principally through attachment to the host cells (Howard et al. 1987; Thomas et al. 1991; Sachse et al. 1993a), cell injury as the result of production of toxic substances such as a complex polysaccharide (Geary

et al. 1981; Rosenbusch 1996), nucleases (Minion and Tigges 1990), lymphokines lymphotoxin (Bennett and Jasper 1978a), toxic oxygen radicals (Thomas et al. 1986; Tryon and Baseman 1992) and haemolysins (Gourlay and Howard 1979), deprivation of nutrients from, or alteration of metabolic activity of, the host cells (Howard et al. 1987; Simecka et al. 1992), immunological responses by the host (Howard et al. 1987) and immunosuppressive properties (Bennett and Jasper 1977a). Also the possibility of entry of *M. bovis* into the host cell, as occurs with *M. penetrans*, *M. pneumoniae* and *M. genitalium* (Baseman et al. 1995) may be a mechanism that enables *M. bovis* to persist in the host cell.

2.9.2 Attachment, cell adherence and invasion

The understanding of the mechanisms of attachment of *Mycoplasma* to host cells assists with recognition of the initial steps of disease pathogenesis and offers a means of treatment based on inhibition of adherence. As *Mycoplasma* are metabolically deficient the close interaction between the membrane of *Mycoplasma* and the host cell probably contributes to survival of *Mycoplasma* by the fusion of the two membranes and transfer or exchange of membrane components. Moreover the adhesion prevents the destruction of *Mycoplasma* by catalase and peroxidase enzymes that are present in mucosal secretions. *Mycoplasma* produces a number of enzymes including phospholipases, proteases and nucleases that probably have a major role in the attachment process (Pollack and Hoffmann 1982; Bhandari and Asnan 1989; Minion and Tigges 1990; Minion et al. 1993). Recently it was suggested that *M. bovis* has a 26-kDa protein that is responsible for adherence to host cells (Sachse et al. 1993b, 1996). *Mycoplasma bovis* can attach to neutrophils and macrophages in the absence of specific antibodies and persist and even multiply on the cell surface (Thomas et al. 1991). It has been suggested that the lack of locally produced IgA may be responsible for persistence and survival of *M. bovis* (Kenny et al. 1992). Immunoglobulin A binds *Mycoplasma* thus preventing attachment and colonisation at the surface of respiratory epithelial cells (Williams and Gibbons 1972; Brunner et al. 1973), but does not permit opsonisation to occur (Nickerson 1985).

2.9.3 Cell injury and cytophatic effects

Mycoplasma can cause direct or indirect cell injury. Various species differ in the degree of cell injury induced and in the severity and type of tissue injury (Araake 1982). In contrast to many other *Mycoplasma* species that live on or in the epithelium of mucosal membranes,

M. bovis can invade the deeper tracheal tissues, infiltrating between the epithelial cells and amassing in the lamina propria, especially in the region of the basement membrane of the epithelium (Howard et al. 1987, Thomas et al. 1987a). The result is a loss of cellular structure of the lamina propria, thereby lifting and detaching the overlying epithelium.

It has been reported that *M. bovis* produces a complex 73 kDa polysaccharide contained within a glycoprotein complex in the cell. Experimental injection of this toxin into the bovine mammary gland produced eosinophilic mastitis (Geary et al. 1981), vascular permeability and complement activation. It has been reported this toxin is not host-cell specific and is active on diverse mammalian cell targets (Rosenbusch 1996). Other toxic products that might cause cell injury are highly reactive oxygen species, free H^+ and OH^- radicals and lymphotoxin (Bennett and Jasper 1977a, Cole et al. 1985b; Thomas et al. 1986). The toxic oxygen and free H^+ and OH^- radicals can be generated during inflammation or normal cellular activity in the presence of oxygen (Eberhart et al. 1982). They cause tissue damage through lipid peroxidation of cellular membrane components, inactivation or denaturation of enzymes, DNA damage, damage to carbohydrates that can alter the cellular receptor function, and also haemolysis of erythrocytes (Eberhart et al. 1982). The lymphotoxin can cause hydrodynamic changes in the host cells (Rosenau and Constantine 1976).

2.9.4 Host responses immunopathology

The host immune response to *Mycoplasma* infection may be protective but also can be ineffective and even have a detrimental effect (Rosenbusch 1987). It has been suggested that *Mycoplasma* have antigens in common with the host, and can incorporate environmental proteins such as antibodies onto their membrane surface (Bradbury and Jordan 1971; Sethi and Brandis 1972; Bredt 1976). *Mycoplasma* might contribute to cell injury through the formation of immune complex "innocent bystander" damage (Washburn et al. 1980a, 1980b). A study of systemic and local immune responses of gnotobiotic calves infected with *M. bovis* revealed that the lung lesions caused by *M. bovis* are partly due to the host's immune response, presumably contributing to the control of infection, and systemic IgG antibody detected after infection (Howard et al. 1986). Nuclease activity has been reported in *M. bovis* (Minion and Tigges 1990). It may increase the chances of genetic alteration of host cells, leading to an autoimmune response (Vincze et al. 1975).

2.9.5 Immunosuppressive properties

One of the putative pathogenic mechanisms of *Mycoplasma* is their role in modulation of the nonspecific and the acquired immune response (Howard and Taylor 1985; Ruuth and Praz 1989; Stuart 1993). Immunosuppressive effects of *M. bovis in vivo* and *in vitro* have been reported in cattle inoculated with viable *M. bovis* (Bennett and Jasper 1977a; Cole et al. 1985b). It is probable that both B-cell and T-cells functions are impaired (Boothby et al. 1987).

Reduced cell-mediated immune activity may be responsible for the animal's inability to provide an effective immune response against *M. bovis* infection, it may predispose animals to secondary infection and perhaps assist the long-term and widespread nature of the typical infection (Berquest et al. 1974; Bennett and Jasper 1978a; Jasper 1982).

The surface adherence of *M. bovis* also has been shown to reduce the *in vitro* killing of opsonised *E. coli* by bovine neutrophils by Howard and Taylor (1983). They further noted that the adherence of ^{35}[S]-labelled *E. coli* to bovine neutrophils was unchanged by pre-incubation of neutrophils with *M. bovis* at concentrations of organisms which inhibited bacterial killing. Based on these results they suggested that the *M. bovis* induced inhibition of bacterial killing involved ingestion, or a subsequent step in neutrophil microbicidal function. More recently, it has also been shown that *M. bovis* reduced neutrophil activity through inhibition of the respiratory burst (Thomas et al. 1991).

2.9.6 Antigenic variability (phenotype variation)

Mycoplasma strains appear to be highly variable in their phenotypes. They can rapidly lose their virulence through passage in artificial media. *M. bovis* has been reported to contain a set of multiple variable surface lipoproteins (VsPs) which represents a newly discovered system of *Mycoplasma* surface antigen variation (Behrens et al. 1994; Rosengarten et al. 1994). The variability of *Mycoplasma* surface antigens may account for changes in virulence which can result in different patterns of disease caused by the same species of organism. This property allows the organism to adapt to different conditions present during the disease process, enabling them to change their adhesive properties to host cell types, and may also enable them to evade a specific immune response (Simecka et al. 1992; Brunham et al. 1993).

2.10 Immunity to *Mycoplasma bovis* Infection

Clinical observations and experimental and field vaccination studies involving *M. bovis* infection suggested degrees of resistance or immunity in some cattle (Jasper et al. 1966; Chima et al. 1980; Jasper 1981). Resistance was also suspected when cattle in infected herds were shedding the organisms without a history of clinical mastitis, or when herds were observed to have many *M. bovis* infected cattle but only a minimal amount of mastitis (Jasper 1981). Studies of *M. bovis* specific immune responses have failed to differentiate between resistant and susceptible animals (Bennett and Jasper 1978a, 1978d).

2.10.1 Humoral immunity

Immunoglobulins may help protect cattle against *Mycoplasma* infection. IgA and IgG concentrations were substantially higher in milk whey from quarters resistant and partially resistant to challenge reinfection than in secretions from susceptible quarters (Bennett and Jasper 1978c). Experimentally, the study of *M. bovis* infection has revealed that IgM is the dominant immunoglobulin for up to 57 days after inoculation of 70 colony forming units (CFU) of *M. bovis*, and then IgG takes over. Daily IgA and IgG production was greater in those quarters in which the infection had resolved than in quarters in which it had not (Bennett and Jasper 1980). Immunity may be based on locally produced IgA and IgG with cellular input. Although a pre-existing leukocytosis of milk will protect the udder from mastitis (Bramley and Dodd 1984), it will not protect vaccinated cows from *M. bovis* infection (Boothby et al. 1988). Induction of neutrophil infiltration by infusion of endotoxin 16 hours before experimental challenge did not reduce the severity, duration of disease or the isolation of *Mycoplasma* (Brownlie et al. 1979).

The presence of serum components and PMN in vaccinated cows does not affect the continuation of infection, or the propagation or spread of *M. bovis* (Boothby et al. 1988). *Mycoplasma bovis* can attach to bovine phagocytes in the absence of specific antibodies, survive and even multiply on the cell surface (Howard et al. 1976b). This property is uncommon, although the ability to multiply on the surface of competent phagocytes has also been reported for several other *Mycoplasma* spp. (Jones and Hirsch 1971; Cole and Ward 1973). In fact, *Mycoplasma* adhering to the surface of neutrophils inhibits cellular function, including phagocytosis of other bacteria (Simecka et al. 1992). The killing of *M. bovis* by

serum is achieved by the alternate complement pathway. The surface of certain strains may be an activating factor for this pathway. The killing of *Mycoplasma* has been examined and IgG_2 was more effective for PMN while IgG_1 and IgG_2 were more effective for macrophages. Neutrophils cannot kill *M. bovis* unless specific antibody is present. IgG_1, IgG_2 and IgM specific for *M. bovis* did not kill *Mycoplasma* on their own (Howard 1984).

2.10.2 Cell-mediated immunity

Mycoplasma may have a suppressive effect on lymphocyte function, as it was found that calves inoculated with *M. bovis* antigens had reduced lymphoblastogenesis, whereas blastogenesis was increased in those calves that received antigen in Freund's complete adjuvant (Bennett et al. 1977). Moreover, there was little increase in lymphocyte stimulation indices when lymphocytes from naturally infected cows were incubated with viable *M. bovis*, and suppression of those indices when incubation was with *M. bovis* supernatant. Addition of viable *M. bovis* with phytohaemagglutinin reduced activity, as compared with phytohaemagglutinin alone (Bennett and Jasper 1978a). Cows recovered from infection had a low blood lymphocyte stimulation index, which has much reduced following reinfection (Bennett and Jasper 1978c).

2.10.3 Vaccination

One milligram of formalin-inactivated *M. bovis* protein in Freund's complete adjuvant was used to immunise cattle by the subcutaneous route at week 0, 2, 4 and by the intramammary route at week six and eight. Vaccinated cows generate specific IgM, IgG_1 and IgG_2 antibody in serum but no IgA. In comparison, cattle naturally-infected with *M. bovis* showed high IgM, IgG_1 and IgG_2 concentrations in the serum but also had specific IgA in serum. Whey from vaccinated cows had specific IgG_1 only (Boothby et al. 1987).

In a challenge study, cows were inoculated to 1.5×10^6 CFU of live *M. bovis* at week 12. Vaccination did not protect against experimental challenge exposure, nor against spread of infection from infected to uninfected quarters (Boothby et al. 1986a, 1987). There is little evidence that immunisation with killed organisms (both systemically and in the mammary gland) will prevent mammary infection with *M. bovis* and pre-existing systemic antibody does not seem to protect against infection. Possibly IgA is required and this isotype is not

stimulated by a killed vaccine (Boothby et al. 1988). IgA is an important factor in the resolution of *Mycoplasma* respiratory infection in humans, as are T lymphocytes (Kenny et al. 1992). Although live vaccines are more effective than killed vaccines in preventing some *Mycoplasma* infections in animals (Foggie et al. 1970; Lloyd and Trethewie 1970) they are not without some hazards (Foggie et al. 1970; Masiga and Mugera 1973; Dyson and Smith 1975).

Some *M. bovis* strains have been thought to be attenuated (including a strain passaged on artificial media for ten years) but when introduced into the udder, they caused severe mastitis (Boothby et al. 1987). High local concentrations of *M. bovis*-specific IgA have been achieved by immunising cattle with a killed antigen preparation by the intraperitoneal and intramammary routes but this also caused peritoneal adhesions (Lascelles et al. 1981). Vaccination may produce an immune response that can result in cellular inflammation. This inflammatory reaction may damage host tissue because of the close association of *M. bovis* with host cells and/or antigenic exchange between host cells and *M. bovis*, but also may actually assist in resolving *M. bovis* infection (Boothby et al. 1988). Effective vaccine strategies should induce protective immunity without inducing unfavourable toxic reactions or adverse immune reactions, including potentiation of disease (Ellison et al. 1992).

2.11 Control of *Mycoplasma* Mastitis

As there is no effective treatment for *Mycoplasma* mastitis, investigators have consistently recommended segregation or culling of infected animals. The choice between culling and segregation will depend upon the numbers of animals involved, the severity of the disease, physical facilities and financial considerations. If animals are segregated, infected animals should be milked last and great care should be exercised to avoid traffic of personnel and equipment from the infected to the non-infected groups. Cows should usually be kept in the infected group until they leave the herd, even if this is several lactations later (Jasper 1977). If such animals are mixed later with uninfected cows, experience has shown that new outbreaks can occur. Rubber gloves are recommended and should be dipped or rinsed with sanitiser between each cow milked.

Disinfection of milking equipment is important. The teat cups should also be rinsed with sanitiser by back flushing, spray washing, or rinsing in buckets. Hand milking of infected

cows may reduce machine contamination, but is often not practical. The chance of introducing *Mycoplasma* mastitis is reduced if purchases are made from herds known to be free of the disease. Calves should neither be allowed to mix with infected cows nor fed infected milk, unless it is pasteurised (Stalheim and Page 1975; Alexander et al. 1985).

2.12 Economic Impact of *Mycoplasma* Infection

All farm animals may suffer from *Mycoplasma* infections, causing significant economic losses worldwide (Manilcff et al. 1982; Razin and Barile 1985). Mastitis is one of the most complex and costly diseases of the dairy industry (Eberhart et al. 1987). It has been estimated that mastitis costs the Australian dairy industry more than $60 million per year (Munro et al. 1984). The impact of *Mycoplasma* mastitis varies with the quality of preventive and surveillance programs instigated as a part of normal dairy operation. Subclinical and clinical mastitis (Gourlay and Howard 1979; Jasper 1981; Ross 1985) results in decreased milk production (Brown et al. 1990), destroys quarters (Mackie et al. 1986; Jasper et al. 1987) and results in the premature culling of cows with refractory mastitis (Brown et al. 1990). Once *Mycoplasma* mastitis enters the herd the cost of control of the disease increases for a number of reasons (Fritzsch and Bornert 1988). The number of calf replacements available may decrease because of arthritis (Alexander et al. 1985) and pneumonia (Taoudi et al. 1985). This disease in calves then hampers the ability of dairy farmers to cull non-profitable animals from the milking herd voluntarily and makes sustained genetic improvement harder to achieve.

2.13 Diagnosis of *Mycoplasma* Infection in Animals

The diagnosis of *Mycoplasma* infection based on clinical signs alone is difficult (Razin 1987; Kenny 1992; McCully and Brock 1992). The clinical disease often develops insidiously, can advance rapidly and often becomes chronic in nature (Stalheim 1983; Razin 1994). It is easily confused with other bacterial or viral infections, although most bacterial infections respond to chemotherapy with improvement in the clinical condition. Thus the failure of sick cattle to respond to treatment could suggest that the pathogen may be *Mycoplasma* (Stalheim 1976). Therefore laboratory diagnosis is very important in the control of *Mycoplasma* infection. Current methods for diagnosis of animal *Mycoplasma* infections are generally based on isolation by cultivation of *Mycoplasma* from clinical samples followed by serological identification of the isolate (Kenny 1992; Simecka et al. 1992).

2.13.1 Culture

Culture remains the primary diagnostic aid for detection of animal *Mycoplasma* infections. *Mycoplasma* are delicate and specimens must be kept refrigerated and delivered to the laboratory within 24-48 hours of collection. The specimens should ideally be obtained from animals at an early stage of the clinical disease (Quinn et al. 1994). *Mycoplasma* are fastidious organisms with many ecological niches *in vivo*. The general principle in culturing *Mycoplasma* is to imitate the ecological niche of the organism as closely as possible. Therefore, there are many different *Mycoplasma* media, each designed to optimise growth of one or a few species. In general, each contains a nutrient-rich base and serum, plus antimicrobial agents to restrain the growth of other bacteria. Although the general formulae are similar for all *Mycoplasma* media, the contents do vary and can substantially affect the success of isolating specific organisms. It is a common experience to find the isolation of fastidious *Mycoplasma* directly from animals to be much more difficult than growing laboratory-adapted stock cultures. Some *Mycoplasma* strains grow in broth, others of the same species may grow on agar. Some strains need repeated blind subculturing in additional *Mycoplasma* media before they will grow *in vitro*, while some strains will grow only in cell culture systems (Simecka et al. 1992).

Since *Mycoplasma* have a propensity for mucosal surfaces, samples obtained are usually from the respiratory tract, the urogenital tract, the joints, the eyes and the mammary gland (Gourlay 1981). Multiple site samples within the host are required. The preferred site within the host should be chosen if known, in addition to as many other sites as possible. Under most circumstances the limit to detection of *Mycoplasma* by culture is 400 CFU/mL of sample (Simecka et al. 1992). Fewer organisms can be isolated if the greatest care is taken but it is generally uneconomical. Thus culture is relatively insensitive, expensive, and false negative results are common (Davidson et al. 1981; Garcia et al. 1986; Nielsen et al. 1987).

2.13.2 Biochemical characterisation of *Mycoplasma*

Identification of *Mycoplasma* isolates as specific species depends upon serological testing using specific antisera prepared against type strains of each species. The number of species is now so large that much work is needed to provide antisera to all species, and economy in their

use is desirable. Preliminary screening of isolates using several biochemical tests will decrease the number of antisera required for identification.

2.13.2.1 Glucose fermentation

Aerobic and anaerobic growth conditions are applied to assess glucose catabolism as determined by acid production. One percent glucose and 0.002% phenol red are added in the *Mycoplasma* broth media (MBM) and the pH is adjusted to 7.8. Control medium lacks only the glucose (Aluotto et al. 1970).

2.13.2.2 Arginine hydrolysis

Arginine hydrolysis differs between the *Mycoplasma* species. It is determined by a change in pH due to ammonia production. Arginine (0.02%) and phenol red (0.002%) are added to the medium and the pH adjusted to 7.4. Control medium lacks only the arginine (Aluotto et al. 1970). Arginine may restrain growth of some *Mycoplasma* (Leach 1976).

2.13.2.3 Urea hydrolysis

The *Ureaplasma* spp. are classified based on the determination of urea hydrolysis and production of CO_2 and NH_3^+, which results in a rise in pH (Shepard and Masover 1978).

2.13.2.4 Genetic characteristics

The guanine plus cytosine content of the DNA is determined by T_m (Marmur and Doty 1962; McGee et al. 1967) or buoyant density (Kelton and Mandel 1969).

2.13.2.5 Phosphatase activity

A sterile 1% solution of phenolphthalein diphosphate sodium salt is added to the MBM at a final concentration of 0.01%. After incubation, the assay is detected by adding 5 N NaOH to the media to a final concentration of 5%. If the *Mycoplasma* produces phosphatase, a red colour develops immediately (Aluotto et al. 1970).

2.13.2.6 Formation of film and spot

Film and spot production is performed on agar surfaces using an agar that contains egg yolk emulsion (Fabricant and Freundt 1967). An agar plate is inoculated with a drop of overnight broth culture and incubated for two weeks at 37°C. The plate is examined at three day intervals for the appearance of a film over the heavily inoculated area. Examination under a dissecting microscope will help detect the crooked appearance of the film and the small black spots in the upper layer of the medium.

2.13.2.7 Reduction of tetrazolium chloride

The test organism is incubated in MB containing 0.02% of 2,3,5,-triphenyltetrazolium chloride under aerobic and anaerobic conditions for seven days at 37°C. A pink or red colour indicates tetrazolium reduction by the organism (Aluotto et al. 1970).

2.13.3 Histopathology

Detection of microscopic lesions in lungs, nasal passages, joints and mammary glands is useful for confirmation of *Mycoplasma* disease. However gross lesions are not common in many *Mycoplasma* diseases and in animals infected with low numbers of *Mycoplasma* these lesions are scarce. Thus, histopathology is insensitive and requires special training to interpret the results correctly. Minor infections are easily overlooked unless many tissues are studied and one is familiar with minor changes in host tissues. Moreover, lesions are usually expressive rather than diagnostic (Simecka et al. 1992). Establishment of a diagnosis based solely on the presence of gross or microscopic lesions may lead to false negative results.

2.13.4 Serology

Growth of a pathogenic strain of *Mycoplasma* in an animal produces antibodies that can be detected by appropriate serological tests. The clinical usefulness of such a test depends on the ability to distinguish healthy animals from animals with the disease. The reliability of this distinction is defined by the sensitivity and the specificity of the assay (Vecchio 1966). Unfortunately serodiagnostic methods for the diagnosis of *Mycoplasma* infections are not well developed because of the antigenic complexity of *Mycoplasma*. Serodiagnosis has two

significant epidemiological and clinical uses; firstly to determine the incidence of new infections by detection of antibodies produced during the infection, and secondly to determine the prevalence of prior infection in the population by testing individuals for antibodies, the deduction being that those that have antibodies have been infected sometime in the past (Kenny 1992).

The standard antibody response in an infectious disease shows three phases; firstly a quick creation of antibodies during weeks one to three of disease, secondly a peak in antibody concentrations some two to four weeks after infection, and finally a slow decrease in antibody concentration in the months to years following recovery from the disease.

2.13.4.1 Types of serological tests

There are four major serodiagnostic test types for *Mycoplasma*. The first measures the capability of antiserum to prevent growth or prevent the production of a metabolic product used as an indicator (Clyde 1964; Taylor-Robinson et al. 1966). In the second an antigen extract of whole *Mycoplasma* is used and antibodies are detected by the complement fixation test (CFT) (Stalheim 1984), dot blot test (Cumins et al. 1990) and/or enzyme linked immunosorbent assay (ELISA) (Boothby et al. 1981). The third type is the use of a partially purified lipid antigen in an ELISA or CFT (Kenny and Grayston 1965). The fourth type of test is the use of western immunoblots to determine and analyse polypeptides involved in the immune response (Costas et al. 1987; Khan et al. 1987).

2.13.4.2 Metabolic inhibition tests

The growth of the organisms in the presence of known antiserum is measured by the capability of the organisms to produce a metabolic product such as acid from glucose, or failing to reduce tetrazolium or produce ammonia from arginine or urea respectively. The reliability of the assay for discrimination of *Mycoplasma* species is low because it is dependent on the purity of strain, the indicator used and whether the particular species actually utilises these substrates (Kenny 1992). Also great care must be taken to make sure the organism is growing sufficiently in the broth to actually change the indicator. The presence of antibiotic in the serum may inhibit the growth of *Mycoplasma* and produce false negative

results (Busolo et al. 1983). The assay is simple, however the specific requirements of *Mycoplasma* may make it impractical.

2.13.4.3 Complement fixation test

The CFT is one of the most widely applied of all serological techniques (Mori et al. 1983; Stalheim 1984). It has been recommended by the Office Internationale Des Epizooties for diagnosis of *Mycoplasma* infections (Regalla 1995). The test relies on the ability of an antigen-antibody complex to fix complement. The reaction is visualised by the failure of sheep erythrocytes treated with haemolysin (the secondary system) to lyse in the presence of a test serum. When the test serum reacts with the antigen it removes the complement from the system making it unavailable for use in the secondary system. Although this serological test is the most reliable method currently available, it has some major shortcomings in sensitivity and specificity (Jones et al. 1989; Regalla 1995). Only IgM and some IgG subclasses are detected by this method (Busolo et al. 1983); moreover the assay is very complicated, particularly regarding the standardisation and preparation of the required reagents.

2.13.4.4 Immunofluorescence and immunoperoxidase

These methods have been used to detect *Mycoplasma* antigen in tissue samples (Davidson et al. 1983; Hill 1983; Jordan 1983; Ogata 1983; Razin 1985). Although both methods work well in animals that are heavily infected, they demand special processing of tissues and trained personnel to interpret the results. In animals infected with 10^3 CFU or less, immunofluorescence (IF) detected less than 50% of infected animals, and even this grade of success required examination of various tissues. Examination of an individual tissue gave a much lower success rate (Simecka et al. 1992). Discrimination of *M. bovis* from *M. agalactiae* by immunoperoxidase (IP) and IF is impractical because these two types share the property of autofluorescence and other biochemical characters (Erno and Stipkovits 1973; Leach 1973; Poumarat et al. 1991). Moreover, they show as bright yellow, that hampers their identification using IF (Potgieter and Ross 1972; Polak-Vogelzang et al. 1978; Poumarat et al. 1991).

2.13.4.5 Enzyme-linked immunosorbent assay

One of the most important assays performed in veterinary medicine is the ELISA. It can be used to detect and quantify either antigen or antibody present in animal sera. In its simplest form, the ELISA is performed by allowing *Mycoplasma* antigens to bind non-specifically to a solid phase (usually a 96-well microtitre plate) then washing to remove unbound antigen. The serum to be tested is added to the plate and specific antibodies in the serum bind to the antigen. After incubation and washing to remove unbound antibodies, the presence of bound antibodies is detected by addition of secondary conjugated antibody, and enzyme substrate. Adequate washing between additions reduces non-specific binding that results in false positive reactions. The assay is completed by developing the coloured product of the enzyme reaction, and the colour change is read either on a spectrophotometer or by visual inspection (Liberal and Boughton 1992).

The ELISA can detect *Mycoplasma* specific IgM, IgA and IgG in serum and whey, therefore an antibody response profile of infected animals can be determined (Horowitz and Cassell 1978). This information enables an estimation of both the immune status of infected animals and the competence of vaccine preparations in eliciting a protective response. Several ELISA assays for detecting serum antibodies against bovine *Mycoplasma* and *Ureaplasma* spp. have been reported. Boothby et al. (1981) developed an ELISA to detect *M. bovis* IgG in bovine serum. Cross reactivity of bovine serum with *Mycoplasma* antigens of bovine (*M. bovis, M. bovirhinis and M. californicum*), caprine (*M. agalactiae*), avian (*M. gallisepticum*), and environmental sources (*Acholeplasma laidlawii*) have been tested. Crossreactions have been reported in all instances, with the strongest cross reaction between *M.bovis* and *M. agalactiae*.

2.13.4.6 Western blots

The Western blot was originally described by Burnette (1981) and Towbin et al.(1979). It is the most common analytical method commonly used to identify a specific target antigen in an organism (Towbin et al. 1989). Proteins isolated from the test organism are separated using polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Strips are cut from nitrocellulose membrane and used to assay animal sera for antibodies by immersing the nitrocellulose into different dilutions of the serum. After washing, bound antibodies are detected with an enzyme-conjugated antibody to the target immunoglobulin.

The enzyme label is detected using a histochemical stain that precipitates on the nitrocellulose and identifies the molecular weights of reactive polypeptides. The assay has been widely used to detect the antigen profile of *Mycoplasma* isolates (Costas et al. 1987; Khan et al. 1987; Thirkell et al. 1991; Sachse et al. 1992), but it has essential limitations in its application because of antigenic variation, low sensitivity (10^6 CFU/mL), the impracticality of direct detection of organisms in clinical samples, and the cost and time required (Sachse et al. 1992).

2.13.4.7 Monoclonal antibodies

The advent of hybridoma technology for production of MAbs has made it feasible to produce large amounts of identical antibodies, specific for particular antigens, that can be used in research, diagnosis, species typing, and epidemiology. Monoclonal antibodies are now widely used in detection of *Mycoplasma* (Olson et al. 1991; Feld et al. 1992; Panangala et al. 1992). Jurmanova et al. (1986) described the use of an ELISA for the detection of antibodies against *Mycoplasma* spp. and *Ureaplasma* spp. in milk. ELISA procedures using MAbs have been used to detect *M. bovis* antigen in semen and preputial washings (Nielsen et al. 1987), and in milk (Boothby et al. 1986b). The assay described by Boothby et al. (1986b) allows the detection of 10^7 CFU/mL with no cross reactivity with other bovine *Mycoplasma* species including *M. californicum, M. alkalescens, M. bovigenitalium, M. canadense, M. bovirhinis, M. arginini,* and *Acholeplasma laidlawii.* Using an immunobinding assay involving the same MAb, the sensitivity of the assay was increased to 4.8-5 x 10^3 CFU/mL milk (Infante et al. 1989). Due to difficulties regarding automation, immunobinding assays have not found popular use in routine diagnosis.

Ball et al. (1990) described an antigen capture ELISA involving a MAb to detect *M. californicum* in milk samples. Using centrifugation to enrich the antigen, the sensitivity of this test was improved from 10^7 to 10^6 CFU/mL milk. Also, to increase the sensitivity of the assay the plates were coated with polyclonal antiserum and the MAb was labelled with fluorescence. Although this resulted in a reduction of background by 75%, the sensitivity of the assay was not enhanced because alkaline phosphatase intensities of positive samples were reduced by a similar extension. A fluorescence ELISA using a MAb for the detection of *M. bovis* antigen in milk has been assessed (Heller et al. 1993). Milk samples were pre-enriched by mixing equal volumes of the samples with MBM and incubating for 48 hours. The sensitivity of this test was 10^5 CFU/mL of milk, but cross-reaction with *M. agalactiae*

occurred. The attainable sensitivity of these antigen detection ELISAs are insufficient for the reliable identification of animals shedding *M. bovis*. Moreover, the application of an antibody detection ELISA is limited by the fact that peak antibody titres occur ten to fourteen days after the onset of disease (Sachse et al. 1993a).

2.13.4.8 Problems with serological tests

Serological methods are extensively used for the detection of *Mycoplasma* infections, but they are generally non-specific, relatively insensitive and depend on the competence of animals to produce specific antibody (Clyde et al. 1984; Ewing et al. 1996). They require increased antibody titres that are only reached seven to 10 days after the beginning of clinical signs. Moreover, the organisms may evoke only low serum antibody titres (Feenstra et al. 1991) following respiratory infection in very young or very old animals, and in T- and B-cell defective animals. Also, there may be a delay in antibody production with a subclinical infection, resulting in a 1-3 month time lag during which infected animals cannot be detected by serological methods (Simecka et al. 1992).

Detection and identification of *Mycoplasma* directly from clinical samples by conventional serological methods are restricted. *Mycoplasma* incorporate components of the growth medium in their membrane (Bradbury and Jordan 1971; Nicolet et al. 1980; Thorn and Boughton 1980) and when used as antigens for the production of specific antiserum, they raise antibodies to growth medium components and *Mycoplasma* antigens. Furthermore, many *Mycoplasma* are not strong immunogens and do not elicit profound and specific antibody responses (Morton and Roberts 1967). *Mycoplasma bovis* infection may or may not be accompanied by an antibody response (Bennett and Jasper 1978a; Jasper 1981). Conventional antisera show considerable variation due to the method of antigen preparation and administration, and responsiveness of individual animals used in the preparation of antisera. This situation makes detection by serological methods very difficult. The major problem for discrimination between *Mycoplasma* species is cross-reactions (Lemcke 1973; Thirkell et al. 1991; Abdelmoumen and Roy 1995). These may be particularly problematic when trying to discriminate between *Mycoplasma* species. All of the improved methods, including the development of ELISA, employment of immunoblots to confirm ELISA results, blocking of cross-reactive antibodies with rabbit antisera, precipitation of cross-reactive antibodies with heterologous organisms, and the removal of cross-reactive antigens by bond chromatography

to rectify species specificity have proven difficult to optimise (Simecka et al. 1992). Also using specific anti-*Mycoplasma* IgM does not improve rapid diagnosis of *Mycoplasma* infection, because sera would optimally need to be collected within a week after the onset of the disease (Vikerfors et al. 1988).

2.14 Molecular Methods for Detection of *Mycoplasma* Disease

2.14.1 Introduction

Presently, there is no thoroughly satisfactory diagnostic test for *Mycoplasma* infection in animals applicable to all situations. The combination of diagnostic tests chosen must be adapted to the desired plan, and the results must be interpreted with care. An assay adequately sensitive to detect low level *Mycoplasma* infection needs to be developed. Two candidates that seem practicable with current technology are specific *Mycoplasma* antigen detection assays and the PCR using specific primers (Simecka et al. 1992; Kirk and Lauerman 1994).

During the last 10 years there has been a revolution in methods for the diagnosis of infectious disease, mainly as a result of improvement of techniques in molecular biology. In view of the potential of these methods, it is widely agreed that DNA-based diagnosis of *Mycoplasma* disease is emerging as the diagnostic tool of choice for the future. The aim of diagnostic microbiology is to supply methods by which a specific pathogen can be quickly detected within a clinical sample in enough time to have an impact on the care and treatment of the diseased animal (Falkow 1985). The great difficulties in culturing *Mycoplasma* and serodiagnosis have already been discussed. Recent research reveals that many pathogenic *Mycoplasma* grow very slowly, if at all, in the currently available media thus hampering laboratory diagnosis of *Mycoplasma* infections (Razin 1994). Growing *Mycoplasma in vitro* by copying the natural environment provided by the host is very difficult. Thus the development of a DNA-based diagnosic method specific for *Mycoplasma* offers great hope for rapid, sensitive, specific and correct diagnosis of *Mycoplasma* infection (Razin et al. 1987). The simplicity of the DNA hybridisation procedure, in combination with an advanced detection system, is well suited for processing large numbers of samples and with different genome projects gathering speed, DNA hybridisation and detection has now become a major focus for automation (Beck 1995).

2.14.2 DNA hybridisation

The development of DNA hybridisation techniques for the quick diagnosis of microbial infections constitutes a new advance for solving the problems involving the detection of fastidious organisms including *Mycoplasma* (Hyman et al. 1987; Khan et al. 1987; Razin et al. 1987; Santha et al. 1987). The technique was first described by Hall and Spiegelman (1962) and now has widespread application.

The principal of the DNA probe is based on the capability of two complementary DNA strands to form a double helix or hybrid which is held together by hydrogen bonding. The DNA of target organisms can be cloned into a plasmid vector, which is easily maintained and produced in large quantities within the host, usually *E. coli*. By labelling one strand that subsequently is used as the probe in the hybridisation, the complementary strand or target can be identified with great specificity. Clones carrying DNA fragments of the target organisms are screened for fragment size and then tested for cross reactivity with other bacteria likely to be present in the biological samples (Hyman et al. 1987). Most importantly, the probe must be specific for the targeted organism to eliminate false-positive responses, while keeping enough sensitivity to detect early infections and therefore speeding up the diagnosis.

The general diagram for DNA hybridisation and detection is shown in Figure 2.2. All hybridisation strategies include the same four steps: prehybridisation (to block non-specific probe binding sites), hybridisation, washing and detection.

DNA probes for the detection of *Mycoplasma* have several advantages over conventional methods. They can detect targets that ordinarily demonstrate antigenic variation, they have high specificity and sensitivity, the marked stability of DNA enables the testing of dried samples (a great advantage in epidemiology studies), and importantly they can detect *Mycoplasma* infection before the appearance of a specific serological response (Falkow 1985).

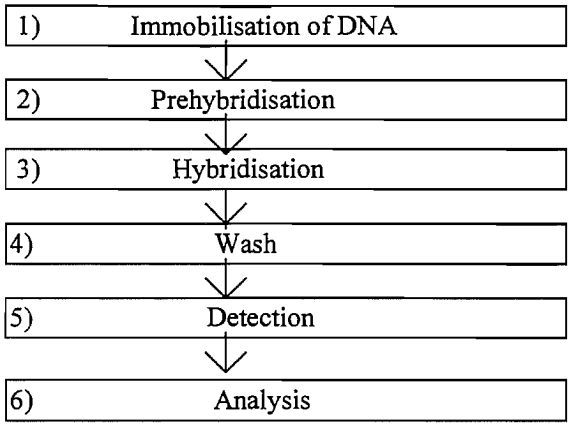

Figure 2.2 General procedure for DNA hybridisation and detection.

2.14.2.1 Types of DNA probes

To date, there are three types of DNA probes. The first are probes made of rRNA genes or fragments thereof (Razin et al. 1984). Due to the highly conserved nature of rRNA genes, probes of this type demonstrate low specificity. The second kind comprises *Mycoplasma* DNA fragments composed of sequences specific for certain species or strains (Taylor et al. 1985). The third type utilises extrachromosomal DNA, such as *Mycoplasma* plasmid or viral DNA as the probe (Razin et al. 1987).

2.14.2.2 Sensitivity and specificity of DNA probes

DNA probes for many species of *Mycoplasma* have been developed (Hyman et al. 1987; Hyman et al. 1989; Dedieu et al. 1992; Zhao and Yamamoto 1993). The efficiency and kinetics of DNA hybridisation depend upon various parameters such as the buffer composition (pH, ionic strength), the hybridisation/wash temperature, and the properties of the probe such as its length and base composition and whether is single or double stranded (Meinkoth and Wahl 1984; Hames and Higgins 1985). Improvement in labelling of the DNA probes, finding the optimal ratio of test to probe DNA, checking for the best hybridisation conditions, and determining trivial factors in detection such as size of dots and exposure time of the film all have a great influence on sensitivity of DNA probes. Increased conditions of stringency enhance specificity of the probe and decrease sensitivity (Razin et al. 1987). Recently, the detection of *M. bovis* using an oligonucleotide probe prepared from partial

sequences of *M. bovis* 16 S rRNA (Mattsson et al. 1991) and a probe prepared from 1150 bp of genomic *M. bovis* DNA (McCully and Brock 1992) have been reported. They lacked specificity however because of cross-reactivity with *M. agalactiae* and *M. arginini*, respectively. Comparison of the nucleotide sequence of the 16S rRNA gene of *M. bovis* with 16S rRNA sequences of *M. agalactiae* showed that they differ at eight nucleotides only (Mattsson et al. 1994).

2.15 Polymerase Chain Reaction

The PCR is a powerful technique which can selectively amplify a specific segment of DNA from a complex mixture of other nucleic acids (Weiner et al. 1995). The introduction of PCR has superseded the previously developed DNA probes by providing faster and much more sensitive tests. The method was used originally to diagnose genetic disease and viral infection (Saiki et al. 1986). Polymerase chain reaction is an *in vitro* technique for amplification of short segments of DNA and involves the use of two oligonucleotide primers that flank the DNA segment to be amplified. Repeated cycles of heat denaturation of the DNA allow primers to anneal to their complementary sequences, and the annealed primers are extended using DNA polymerase. These primers hybridise to opposite strands of the target sequence and are oriented such that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Moreover, since the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles DNA synthesised in the previous cycle. This results in the exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles (Saiki et al. 1988). The reliability of PCR is most often compromised by the occurrence of contamination, leading to false positive results.

2.15.1 Sensitivity of PCR

Polymerase chain reaction is an effective tool for bacteriological diagnosis and is more sensitive and reliable than conventional methods (Buck et al. 1992; Skakni et al. 1992; Tola et al. 1996). It can theoretically detect even a single organism (or genome equivalent) in infected tissues. The ability to potentially detect a single *Mycoplasma* cell makes PCR the most sensitive detection method available, even more than cultural methods. A positive culture can be derived from a single *Mycoplasma,* however in practice, due to many reasons, a positive

Mycoplasma culture requires an inoculum equivalent to about 100 to 1000 cells (Buck et al. 1992; Skakni et al. 1992; Marmion et al. 1993; Tenover and Ungar 1993). Infection in asymptomatic animals and animals treated with antibiotics may also be detected with the PCR procedure. Animals can be detected as positive by PCR earlier than the serological response to the organism, and infection can also be detected in an immunocompromised host (Skakni et al. 1992). The PCR may not be affected by the presence of DNA of other bacteria in the clinical specimen. It is a common finding that faster growing bacteria will overgrow and mask, or inhibit growth of the fastidious *Mycoplasma*, even when appropriate media are used (Van Kuppeveld et al. 1993). Nevertheless, false-negative PCR results and false positive results are rather common in the PCR assay due to inhibitors in clinical specimens and contamination of reagents with target or PCR amplicon DNA (Razin 1994).

A PCR for the detection of *M. bovis* has been described (Hotzel et al. 1993, 1996) but the sensitivity of the assay is low and the detection of PCR product is complicated.

2.15.2 Development of polymerase chain reaction assay

2.15.2.1 Detection of target sequence

The first stage in development of a PCR is to identify and obtain the sequence of target DNA (Figure 2.3). A reliable technique for finding a unique DNA sequence is by "universal primer PCR amplification" of ribosomal (r) DNA sequences (Medlin et al. 1988; White et al. 1990; Persing et al. 1992). Complete or almost complete sequences of the 16S rRNA genes are now accessible for 50 *Mycoplasma* species, and can be retrieved from several data banks (Johansson 1993). The *Mycoplasma* 16S rRNA genes carry, in addition to the conserved regions, more specific variable regions and specific 16S-23S intergenic spacer regions (Harrasawa et al. 1993a). Primers can be selected from these regions with various degrees of specificity (Robertson 1993). The sequence to be amplified can also be chosen from a published *Mycoplasma* protein gene sequence (Jensen et al. 1989; Buck et al. 1992; Skakni et al. 1992; Cadieux et al. 1993) or from a randomly cloned DNA fragment demonstrated to be specific for the *Mycoplasma* to be detected. The target sequence should be as specific to the microorganisms as desired, i.e. either species or genus specific. To detect all isolates of that species, the sequence should not be polymorphic or demonstrate variability. Stability of sequence is essential for good results in clinical usage. Moreover, genetically unstable

virulence determinants may be lost during primary isolation or in serial passage (Persing 1993). When sequences are present in multiple copies within the organism, for example rRNA genes, repetitive satellite DNA, and kinetoplast mini circle DNA, the analytical sensitivity of the assay will be greater (Razin 1994). Often no suitable DNA sequences are accessible for a particular microorganism using universal primers and therefore they are isolated by screening a genomic DNA library for species-specific sequences and repetitive DNA sequences, using the total genomic DNA as a probe (Wieland et al. 1990).

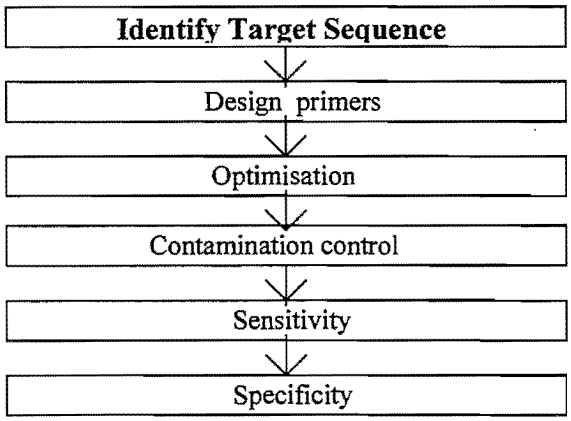

Figure 2.3 Steps involved in the development of a polymerase chain reaction assay.

2.15.2.2 DNA sequencing

Once unique clones are identified their nucleotide sequence should be determined and examined for interspecies heterogeneity. The combination of PCR to amplify genomic DNA fragments (Mullis and Faloona 1987) and an automated DNA-sequencing procedure involving fluorescent dye-labelling terminators to sequence the PCR-amplified DNA fragment directly (Tracy and Malcahy 1991) enables a 300- to 500-bp DNA fragment to be sequenced in 24 hours (Swaminathan and Matar 1993). Each dideoxynucleotide (A, C, G and T) is labelled with a different coloured fluorescent dye, and only one sequencing reaction is carried out containing all four fluorescent dideoxynucleotides. The reaction mix is then electrophoresed on a polyacrylamide gel, which is scanned by a laser as it is running to detect the fluorescent bands. The sequences are automatically determined from the colour of the bands.

2.15.2.3 Primer selection

Direct comparison of genomic DNA sequences of bacterial strains is the best means of quantitatively determining the similarities between two strains. Analysing short segments of DNA for sequence similarities and differences is possible. The correct selection of oligonucleotide primers is very important in setting the performance level of a PCR detection system. Factors to be considered in designing PCR primers include: oligonucleotide length, oligonucleotide temperature, sequence composition, physical characteristics, primer-primer interaction, length of the amplified target and location on the target sequence (Persing 1993). A critical feature of the PCR is the specific annealing of oligonucleotide primers to their DNA target sequence, and their subsequent extension. The design of primers of appropriate sequence is therefore of crucial importance for a successful amplification.

Primers should be designed to have sequences exactly complementary to the sequence to be amplified otherwise mismatches between target and primer will reduce binding efficiency. In addition the primers should also be directed against well-conserved regions of DNA, so that minor genetic differences between target DNA from different strains are less likely to interfere with primer binding. Designing oligonucleotide primers 15-30 nucleotides in length and with a G/C content of 50-60% is preferable (Williams 1989; Innis and Gelfand 1990).

To prevent non-specific primer binding and subsequent amplification of non-target sequences, it is much better to design primers with a high annealing temperature (Rolfs et al. 1992). The best results are obtained using annealing temperatures above 50°C and the annealing temperatures of primer pairs should be matched.

Primers with self complementarity regions should be avoided, as they may form significant secondary hairpin structures, thus preventing their binding to the target sequence (Saiki 1990). Similarly, primer pairs with significant inter-primer complementarity should be avoided. Otherwise this results in the formation of primer dimers that can then serve as template and continue to be amplified at the expense of the desired target sequence (Williams 1989, Rolfs et al. 1992).

There are numerous computer programs, such as OLIGO, to assist in the design of oligonucleotides (Rychlik and Rhoads 1989). These programs can predict melting

temperature, self-complementarity and inter primer complementarity of any given oligonucleotide sequence. Moreover, computer technology can be used to screen primers against sequences in the Genbank and EMBL databases and therefore determine the specificity of the primer for the selected target sequence.

2.15.3 Optimisation of the PCR

After the design of the oligonucleotide primers, the next step is optimisation of the PCR reaction parameters with purified DNA.

2.15.3.1 Reaction buffer

The reaction buffer is designed to optimise Taq polymerase activity. The reaction buffer contains KCl, Tris (pH 8.3), $MgCl_2$, and deoxynucleotide triphosphates (dNTPs) (Saiki 1990). The Tris buffer is necessary for optimal Taq polymerase operation (Sambrook et al. 1989) and the KCl facilitates primer annealing (Innis and Gelfand 1990). The dNTPs are generally added at a concentration of 0.1-0.2 mM each and are added in equimolar amounts regardless of the nucleotide composition of the target sequence (Innis and Gelfand 1990). Variations in concentrations of dNTPs may result in low specificity and poor PCR product yield. A concentration of Taq polymerase between 0.5-5 U/100 µL PCR reaction should be trialled. High concentration of enzyme results in non-specific priming while low enzyme concentration will produce reduced amounts of product (Persing 1993).

The most critical buffer component is $MgCl_2$.The optimisation Mg^{2+} concentration is very important for each individual PCR protocol (Williams 1989; Persing 1993) as the Mg^{2+} concentration affects many reaction parameters in PCR, including primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimmer artefacts, and enzyme activity and fidelity. Optimal concentration of Mg^{2+} usually falls within the range of 0.5-3 mM (Williams 1989). Other ionic components, such as K^+ and Ca^{2+}, also may influence the yield of the PCR products (Innis and Gelfand 1990). Because of the tendency of $MgCl_2$ solution to precipitate after freeze-thawing, stock solutions of $MgCl_2$ should be kept at 4°C rather than the freezer (Persing 1993).

2.15.3.2 Thermal cycling conditions

The thermal cycle conditions include three temperatures, denaturation, annealing, and extension, corresponding to the three steps in a cycle of amplification. The duration of time for each step of the thermal cycle can also affect the sensitivity and specificity of the PCR (Innis and Gelfand 1990; Hosta and Flick 1992).

The denaturation and extension parameters are usually similar in all PCR assays but the annealing temperature varies between assays. Insufficient heating during the denaturation step can be a common cause of failure in a PCR reaction (Saiki 1990). The DNA extension step is performed at 72°C, which is the optimum temperature for Taq polymerase activity (Innis and Gelfand 1990). If the PCR product is less than 500 base pairs it requires an extension time of only 30 seconds, while products greater in size may require 2 min or longer (Williams 1989). Generally denaturation steps of 30-60 seconds at 94°C are sufficient (Innis and Gelfand 1990). The choice of an appropriate annealing temperature is the most critical parameter, since this has the most pronounced effect on the specificity of amplification for any given primer pair (Rychlik et al. 1990). The annealing temperature depends on the T_m of primer and must be optimised. High annealing temperatures may prohibit primer annealing and as a result, no PCR product will be detected. Low annealing temperatures will result in non-specific reactions that may confuse the interpretation of the results (Innis and Gelfand 1990).

2.15.4 Specificity of PCR

Following the selection and validation of the parameters for PCR and definition of the cut-off for positive and negative reactions it is necessary to determine the specificity of the assay. This determines whether the assay detects and properly recognises strains isolated from a wide range of geographic locations, all known serotypes and also excludes all other related organisms and organisms that might be present in the specimen or that result in a similar clinical presentation (Persing 1993). One method for determining the specificity of the PCR assay is restriction enzyme digestion of the PCR products with one or more restriction enzymes that are then subjected to agarose gel electrophoresis. Restriction enzyme analysis may be used to corroborate the identity of PCR products (Eldadah et al. 1991).

2.15.5 Detection of PCR products

2.15.5.1 Ethidium bromide staining agarose gel

Detection of the results from a PCR reaction requires the selection of a reporter molecule and a detection format. The simplest method of detecting PCR products is electrophoresis in agarose gels (Saiki et al. 1985). The gel contains ethidium bromide, or is stained later with ethidium bromide, and then the bands present are visualised using a UV transilluminator to determine whether the appropriate sized band is present. The assumption made with this simple detection method is that a correct sized product is in fact the amplification product of the desired sequence, and not a non-specific amplification product that is incidentally the same length. Further testing can be performed to confirm the specificity of the PCR product, but provided there are no other non-specific products present, this does not usually have to be carried out. One drawback of agarose gel electrophoresis is that its sensitivity can be limiting. When trying to detect sequences present at very low copy numbers (less than 100), even after an amplification factor of 10^9 by PCR, there may still not be enough DNA generated to be visible on an agarose gel. The test sample may then be falsely scored as negative. Nevertheless, for other uses where extreme sensitivity is not required, agarose gel electrophoresis is the simplest and fastest method for detecting PCR product.

2.15.5.2 Hybridisation

This method involves hybridising the PCR product with a labelled probe designed to bind within the amplified region. Either a Southern blot, or a quicker dot blot format can be used. This method can be used to corroborate the specificity of the PCR product and can also be used to increase the sensitivity of PCR product detection compared with that of agarose gel electrophoresis (Myers and Gelfand 1991; Shimizu et al. 1993). The sensitivity of the hybridisation assay for detecting PCR products is 100 to 1000-fold higher than agarose gel electrophoresis. Historically, radiolabelled probes have been used as a detector molecule because of their inherent high sensitivity. However, non-radioactive probes have several advantages, chiefly their greater stability, which allows for standardising reagents; they are less hazardous to personnel and the environment, and are simpler to dispose of. For the wide-scale adaptation of PCR assays, especially in field trials, it is essential to have a rapid, simple, economical, and robust diagnostic assay. Thus it is necessary to exclude radioactivity,

hazardous materials, reliance on expensive equipment, and multiple laborious steps (Persing 1993).

2.15.5.3 Nested PCR

To increase sensitivity and specificity and remove the hybridisation step with the probe, a nested PCR (two-step PCR) has been developed (Harrasawa et al. 1993a, 1993b; Hopart et al. 1993). In this procedure a sample of the amplification product is subjected to a further round of PCR with a set of primers located within the first set of primers. However, eliminating false-positive reactions is very difficult due to target DNA or PCR amplicon contamination (Van Kuppeveld et al. 1994). Sample contamination that can lead to a false positive results. Sample-to-sample contamination, contamination from environmental sources of target sequence (plasmid DNA, *in vitro* culture, etc.) or contamination with the aerosolised product of previous reactions are all examples of how false positive reaction may occur. In addition to physical containment of amplified material, there are chemical and enzymatic methods which may selectively inactivate amplified DNA (Persing and Chimino 1993).

2.15.6 Sample preparation for PCR

Sample preparation is very important in the PCR assay and it requires the most optimisation to develop a reliable process (Razin 1994). Preparation of biological material for PCR requires lysis and release of the nucleic acids, stabilisation of the nucleic acids, efficient target nucleic acid recovery and the removal of inhibitors of DNA polymerase activity, and other components that interfere with primer binding to target sequences (Greenfield and White 1993). Because *Mycoplasma* have no cell walls, in some cases boiling of the sample in distilled water or PCR buffer following its concentration by centrifugation is enough to release the DNA (Teyssou et al. 1993). However some samples contain inhibitors of the PCR reaction, that reduce the efficiency of amplification so that DNA extraction has to be employed (Horner et al. 1993). For example following the solubilisation of the specimens by sodium dodecyl sulphate (SDS) and proteinase K, *Mycoplasma* DNA can be purified by phenol and chloroform extraction followed by ethanol precipitation (Garret and Bonnet 1993). If the method of sample preparation crudely lyses the organisms and does not purify the DNA or target sequence, then either the target sequence needs to be present at high concentrations (Greenfield and White 1993).

Contaminants, including proteins, haem, polysaccharides, phenol, chloroform, ethanol, EDTA, SDS and high salt concentrations, may inhibit the PCR reaction and lead to false negative results. The efficiency of amplification of DNA by PCR depends on the purity of the DNA template. The effect of PCR inhibitors in specimens to be tested may be abolished, or reduced, simply by the diluting the specimens (Blanchard et al. 1993).

Filtration and target capture procedures allow for analysis of larger volumes of specimen, but they are costly and inefficient and involve multiple steps. The need for DNA purification is a serious drawback that hampers the adaptation of PCR for general use in the clinical laboratory. The selection of the most appropriate sample preparation technique depends upon the microorganisms and the cellular and chemical composition of biological samples in which the microorganism is present (Greenfield and White 1993).

CHAPTER 3

DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF *MYCOPLASMA BOVIS* ANTIBODY

3.1 Introduction

The enzyme linked immunosorbent assay was first designed by Engvall and Perlmann (1972) for the quantitative determination of antibodies to bacterial, viral and parasitic pathogens. It has also been used for the serodiagnosis of *M. pneumoniae* in humans (Busolo et al. 1980), *M. hyopneumoniae* in pigs (Bruggman et al. 1977, Djordjevic et al 1994), *M. pulmonis* in mice (Horowitz and Cassell 1978) and *M. mycoides* in cattle (Onoviran and Taylor-Robinson 1979).

In comparison with the CFT and metabolic inhibition tests, that fail to detect antibodies to *Mycoplasma* under certain conditions readily, ELISA is a sensitive and broadly reactive assay for the detection of antibody to *Mycoplasma* (Davidson et al. 1981; Busolo et al. 1983). It can measure IgM, IgA and IgG that may develop against *Mycoplasma* in the serum of animals (Boothby et al. 1981; Busolo et al. 1983). Bruggman et al. (1977) showed that ELISA could detect antibody to *M. hyopneumoniae* five weeks before and 50 weeks after the clinical manifestations of experimental enzootic pneumonia in specific-pathogen-free pigs. Similar sensitivity has been shown for detection of *M. pulmonis* infections in mice (Horowitz and Cassell 1978).

In view of the known sensitivity of the ELISA, an indirect ELISA was developed to evaluate *M. bovis* antibody in animal and human sera for use in the manufacture of *Mycoplasma* media used through this study. The assay was also used to determine the optimal concentration of reagents used in the production of MAbs reported in a subsequent chapter.

3.2 Materials and Methods

3.2.1 *Mycoplasma* strains

Mycoplasma bovis and *M. arginini* cultures were obtained from the Department of Primary Industry's Veterinary Laboratory at Oonoonba, Townsville, north Queensland.

3.2.2 Preparation of antigens

3.2.2.1 ELISA antigen

Each *Mycoplasma* strain (*M. bovis* and *M. arginini*) was purified by serial selection three times on *Mycoplasma* agar (MA). A single colony was inoculated into 100 mL *Mycoplasma* broth (MB) (Chapter 4) and incubated until a rapidly growing culture was obtained. The growing culture was inoculated into one litre of MB, incubated at 37°C for one week with constant agitation. The seed stock used was checked for sterility by subculture onto blood agar, MacConkey agar and MA (Chapter 4). After incubation the *Mycoplasma* were harvested by centrifugation at 20000 *g* (Suprafuge 22 HFA, 22.50; Heraeus Sepatech GmbH D-3360, Osterode) for 30 minutes at 4°C, followed by three successive washes with *Mycoplasma* wash solution (MWS) (Appendix 1.1). Each wash was performed with a volume of MWS equal to the amount of initial *Mycoplasma* culture medium. It was then washed once with glycine buffer (Appendix 1.2) and resuspended in phosphate buffered saline (PBS). The whole cell antigen was disrupted by five successive freeze-thaw cycles using liquid nitrogen, and 5 x 1 minute sonication cycles (Unicamp) (Berthold et al. 1992). The protein content was then adjusted to 0.5 mg/mL by a spectrophotometer (Pharmacia, LKB, England; Ultrospec III) measuring the optical density (OD) 280 (Harlow and Lane 1988), aliquoted and stored at -20°C.

3.2.2.2 Vaccine antigen

For polyclonal antibody preparation, a single colony of each *Mycoplasma* strain (*M. bovis* and *M. arginini*) was grown in 100 mL of rabbit broth (Appendix 1.3) and then the growing culture was inoculated into one litre (10 x 100 mL) of rabbit broth. The culture was shaken at 37°C for one week, then harvested as described in Section 3.2.2.1. The seed stock used was checked for sterility by subculture onto blood agar, MacConkey agar and MA. The pellet was resuspended in PBS (pH 7.4) (Appendix 1.4) to give an approximately 20-fold concentration, aliquoted in 1 mL samples and stored at -20°C.

3.2.3 Production of polyclonal antibody

3.2.3.1 Immunisation protocol

Polyclonal antibody was produced to determine the optimum concentration of antigen for the indirect ELISA, and for the development of a monoclonal blocking ELISA. It was produced by immunisation of a young adult New Zealand white rabbit using antigen harvested from rabbit broth cultures, as described in Section 3.2.2.2. One millilitre of antigen (from Section 3.2.2.2) was lysed (as described in Section 3.2.2.1) and the protein content was adjusted to 0.5 mg/mL as measured spectrophotometrically. The sonicated antigen was mixed with 1 mL Freunds complete adjuvant and then 1 mL of the emulsified antigen was injected intramuscularly into the thigh muscle of each flank of the rabbit. After two weeks the rabbits were then inoculated at weekly intervals for a further 11 weeks with 1 mL of antigen emulsified with incomplete adjuvant. One millilitre of blood was taken from the ear at weekly intervals to determine the antibody titre using the indirect ELISA, and when necessary the rabbit was further boosted by intravenous inoculation. Serum from several bleedings was pooled and fractionated.

3.2.3.2 Purification

Ammonium sulphate precipitation

Ammonium sulphate was used to precipitate the immunoglobulins from the rabbit serum following the method of Harlow and Lane (1988). A 20-mL volume of immunised rabbit's serum was centrifuged at 3000 *g* for 30 minutes (Suprafuge 22 HFA, 22.50) and the supernatant was transferred to a 250-mL flask and stirred using a magnetic stirrer. Saturated ammonium sulphate was added drop-wise at room temperature over a period of 30 minutes to a final concentration of 50% while the mixture was being gently stirred. The resultant solution was held at 4 °C overnight then centrifuged at 3000 *g* (Suprafuge 22 HFA, 2250) for 30 minutes. The supernatant was removed and the pellet was resuspended in 10 mL PBS. This was dialysed overnight with three changes of cold PBS and then further dialysed overnight in Tris buffer (pH 8.0) (Appendix 1.5) at 4 °C, to remove any excess ammonium ions.

Diethylaminoethyl affigel blue chromatography

A diethylaminoethyl (DEAE) affigel blue column (Bio-Rad, California; Cat. No. 153-60 48) was used to purify the rabbit antibodies. This procedure was carried out at room temperature following the manufacturer's recommendation. The column was first washed with five bed volumes of regeneration buffer (Appendix 1.6) and then equilibrated with three bed volumes of Tris buffer (pH 8.0) (Appendix 1.5). The serum was loaded into the column and fractions of approximately half the volume of the serum which was loaded initially were collected. The column was loaded with another two bed volumes of Tris buffer (pH 8.0) (Appendix 1.5) and two fractions were collected. The column was cleaned with three bed volumes of Tris buffer (pH 8.0, 1.4 M NaCl) and re-equilibrated with another three bed volumes of Tris buffer (pH 8.0) (Appendix 1.5) and stored in the refrigerator. The collected fractions were screened for activity by indirect ELISA. The appropriate fractions were then pooled, dispensed in 1 mL amounts and stored at -20°C.

3.2.4 Optimisation of ELISA

The indirect ELISA was carried out by the method of Engvall and Perlmann (1972) with some modifications. Briefly, the sonicated *M. bovis* antigen (0.5 mg/ml) and unsonicated antigen were diluted 1/100 in antigen coating buffer (Appendix 1.7) and used to coat U bottom microtitre plates (NUNC, Microtitre Plate 96 U bottom well). A 200-µL volume of 1/100 diluted antigen was added to each well of the first column of the plate. To the rest of the columns of the plate 100 µL of coating buffer was added to each well. The antigen was titrated across the plate by doubling dilutions. From the first column 100 µL was transferred to the second and so on across the plate to the last dilution, from which 100 µL was discarded. The plate was sealed and incubated at room temperature overnight to allow the antigen to passively adsorb to the plate. The adsorbed antigen was washed three times with PBS Tween 20 (PBST) (Appendix 1.8), using an automated washing device (NUNC). The plate was then tapped dry. The wells of the first row were refilled with 100 µL of the rabbit polyclonal antibody diluted in Tris di-sodium ethylenediaminetetra-acetate sodium chloride Tween 20 casein (TEN-TC) buffer (Appendix 1.9). Only 50 µL of TEN-TC were added to the remaining rows. The antiserum was titrated by transferring 50 µL from the first row to the second and so on across the plate. The plate was incubated at room temperature for one hour. The unbound antibody was removed by washing the plate with PBST as described above.

Affinity purified goat anti- rabbit IgG conjugated to horseradish peroxidase (HRPO) (Bio-Rad; Cat No.172-1013) was diluted 1/2000 in TEN-TC. Aliquots of 50 µL were added to each well and the plate incubated for one hour at room temperature. The plate was again washed three times with PBST to remove unbound conjugate and then tapped dry. Aliquots of 100 µL freshly prepared of ELISA substrate solution (Appendix 1.10) were added to each well and the plate was incubated for another hour at room temperature. In each test there were two controls, a conjugate control consisting of antigen without serum, and conjugate with substrate. The conjugate controls provided a measure of non-specific binding of conjugate to the antigen, while the substrate control was used as a blank in the spectrophotometer reading. A green colour represented enzyme activity. The OD was read at the dual wavelengths of 415 and 490 nm using a spectrophotometer plate reader (Pharmacia; Ultrospec III).

3.2.4.1 Non-reactive

The serum from rabbit blood collected before making the polyclonal antibody was used as the non-reactive control serum (NRC). The NRC serum was tested in the same plate by considering one row of the plate as the negative control and the same procedure as described in Section 3.2.4 was carried out.

3.2.4.2 Optimisation of conjugate

A checkerboard titration was carried out to find the working dilution for conjugate. Two plates were coated with a fixed amount of sonicated *M. bovis* antigen, at a concentration of 1/800 in coating buffer. The plates were incubated overnight and then washed as described in Section 3.2.4. The polyclonal antibody was titrated vertically from 1/100 in TEN-TC and the plates were incubated for one hour at room temperature. The plate was then washed three times, tapped dry and 100 µL of 1/1000 dilution of goat anti rabbit HRPO conjugate (Bio-Rad; Cat. No.172-1013) was added to the first column and titrated horizontally across the plate. The rest of the procedure was as described in Section 3.2.4.

3.2.5 Evaluation of animal sera for the preparation of *Mycoplasma* media

After optimisation of the indirect ELISA with polyclonal antibodies the assay was carried out on sera prior to its use in media to determine whether it contained *Mycoplasma* antibodies. All the incubation and washing steps were carried out as described in Section 3.2.4 with a few exceptions described as follows. Aliquots of 100 µL of serum from animals (pig, cattle, horse) and human serum were added to the first column of a *M. bovis* antigen coated plate and doubling dilutions were made in TEN-TC up to column 12. The plate was incubated at room temperature and washed as described in Section 3.2.4. Depending on the animal serum, the appropriate HRPO conjugate (goat anti-pig, goat anti-bovine, goat anti-horse, and goat anti-human) was used. The optimal dilution of each conjugate was determined by checkerboard titration. The ELISA substrate reaction was carried out according to Section 3.2.4 and absorbance was recorded.

3.3 Results

3.3.1 Antigen optimisation

The optimal concentration of each antigen was determined by testing duplicate dilutions of reactor and non-reactor control serum. It was found that the use of pig serum in the *Mycoplasma* media produced a non-specific background reaction. Thus, foetal calf serum (FCS) was included in the medium used to prepare the ELISA antigen. Furthermore, the antigen was treated with glycine buffer (Appendix 1.2). These two procedures eliminated the non-specific background reaction.

The polyclonal rabbit serum (PRS), and FCS as a non-reactor control, proved suitable for the preliminary evaluation of the ELISA. The working dilution of unsonicated antigen was 1/400, and 1/800 for sonicated antigen. This suggested that the sonicated antigen was more effective for coating plates. Moreover with the sonicated antigen the conjugate could be diluted further, providing a more economical test. Sonicated antigen diluted to 1:800 (200 ng/well) resulted in satisfactory antibody binding without significant loss of absorbance. Therefore this concentration of antigen was used for coating plates in subsequent assays. The optimal dilution of PRS was 1/2000 when used with the sonicated antigen. The ELISA results could not be confidently interpreted without suitable controls, because of plate to plate variation.

3.3.2 Conjugate optimisation

Conjugate at a dilution of 1/2000 provided maximum discrimination between *M. bovis* reactor and non-reactor sera when sonicated antigen was used to coat the ELISA plate.

3.3.3 Reproducibility of the assay

The reproducibility of the indirect ELISA was determined by retesting the same reactor and non-reactor sera within days and between days. The coefficient of variation of OD values obtained within days and between days were similar and linear, giving excellent reproducibility. After the optimisation of the indirect ELISA, the sera from different animals were tested for preparation of *Mycoplasma* media.

3.3.4 Animal sera

A large number of sera from different animals (pig, horse, cattle and humans) had showed antibodies against *M. bovis* and *M. arginini* as detected by the indirect ELISA (Figure 3.1 and Figure 3.2).

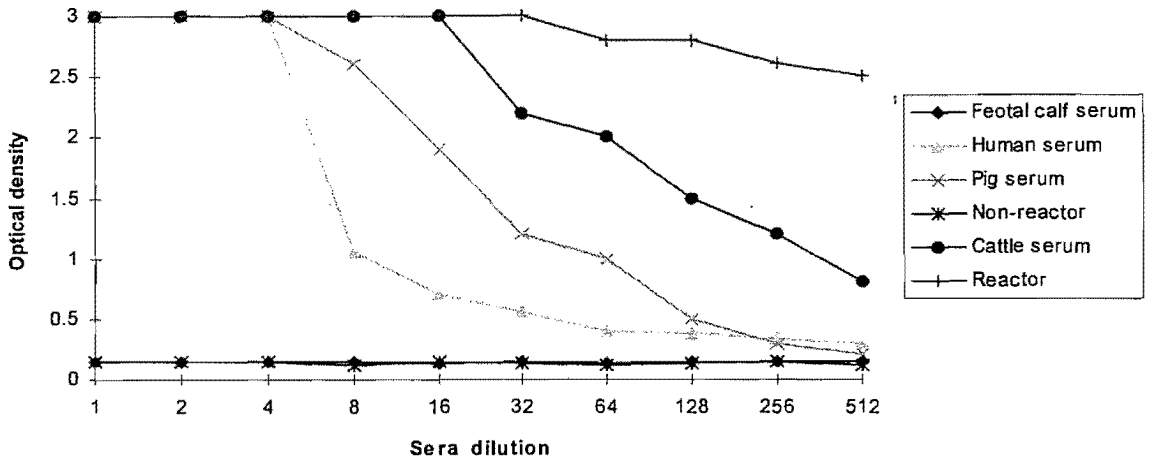

Figure 3.1 The reactivity of animal sera with *Mycoplasma bovis* sonicated antigen in an indirect enzyme linked immunosorbent assay.

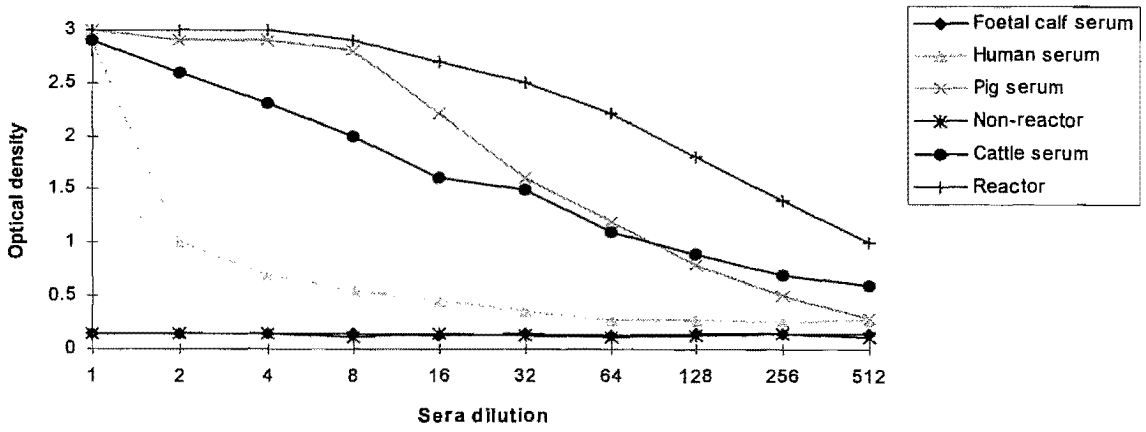

Figure 3.2 The reactivity of animal sera with *Mycoplasma arginini* sonicated antigen in an indirect enzyme linked immunosorbent assay.

3.4 Discussion

The sensitivity and specificity of ELISA depend on the purity of the reagents used (Payment et al. 1979). In this study, the *Mycoplasma* strain used to immunise rabbits was adapted to grow in broth medium supplemented with rabbit serum to remove the risk of incorporation of serum protein into the cell membranes, and thus the risk of of raising antibody to foreign serum proteins in the immunised rabbit (Bradbury and Jordan 1971).

For optimisation of the test reagents, a checkerboard titration was undertaken and ELISA optima were selected based on numerous replicate tests. The dilution of test sera for use in ELISA depends on the intended use of the test. However it has limitations because of contamination of the *Mycoplasma* antigen with serum proteins acquired during propagation of the antigen strain. As reported by other workers (Bradbury and Jordan 1971; Yaguzhinskaya 1976; Nicolet et al. 1980; Thorn and Boughton 1980; Thirkell et al. 1991) it was found that the use of pig serum resulted in an increase in contaminant protein, producing a non-specific background reaction that could not be removed by washing. Therefore the purity of the ELISA antigen preparation is of paramount importance in obtaining accurate and reproducible results.

In this study, a large number of the sera from different animals had antibodies against *Mycoplasma*. Although these antibodies cannot be considered specific for *M. bovis*, the high reaction (OD=3) and high affinity of the bovine antibodies in the indirect ELISA to the *M. bovis* antigen suggests that some of these antibodies are *M. bovis*-specific and are the result of a present or previous infection with *M. bovis*.

The specificity of the indirect ELISA for the serodiagnosis of *M. bovis* infection was determined using sera from cattle, pigs or horses and humans. Although *Mycoplasma* species are mostly host-specific (Stalheim 1984), in this study other animal species also had positive ELISA results, perhaps indicating antigenic similarities between *M. Bovis* and mycoplasmas infecting those hosts. The cattle serum had the highest reactivity with *M. bovis* perhaps indicative of the specificity of the antibody in this animal species.

The results suggest that under optimal conditions, the ELISA described in this chapter is a robust method for the detection of *M. bovis* antibody in animal sera, and is useful for screening sera for *Mycoplasma* media production. The sensitivity of the ELISA might be

related to the ability of this method to measure antibodies belonging to any immunoglobulin class and reacting not only with glycolipid antigen but also with other antigens of the plasma membrane. It was concluded that, due to the reactivity with sera from non-cattle species, that this indirect ELISA configuration lacked specificity for *M. bovis* and that an alternative strategy would be required.

CHAPTER 4

ISOLATION AND CHARACTERISATION OF *MYCOPLASMA BOVIS* FROM THE RESPIRATORY TRACT OF CATTLE IN NORTH QUEENSLAND

4.1 Introduction

In cattle, respiratory disease is a multifactorial condition to which various viruses and bacteria contribute (Ross 1985). It is a major cause of economic loss, due to animal deaths, reduced weight gains and the cost of treatment (Rodriguez et al. 1996). The recognition of *Mycoplasma* as a causative agent of bovine respiratory infection has prompted frequent cultivation of bovine lung tissues for *Mycoplasma* isolation (Gourlay 1973). Mycoplasmal pneumonia is now recognised as one of the most prevalent diseases of cattle worldwide, and it is believed to be of considerable economic importance (Erno and Perreau 1985; Gourlay et al. 1989; Ter-Laak et al. 1992b). However little is known about its prevalence in the respiratory tract of cattle in Australia. Among the bovine *Mycoplasma* species, *M. bovis* is most commonly associated with respiratory infection (Rodriguez et al. 1996). It can cause pneumonia in gnotobiotic calves (Gourlay et al. 1976; Thomas et al. 1986) and often, acting synergistically with other bacteria, can be an important aetiological agent in outbreaks of respiratory infection (Houghton and Gourlay 1983; Lopez et al. 1986; Gourlay et al. 1989). Furthermore, it has also been speculated that infection of the respiratory tract facilitates the spread of *Mycoplasma* infection to other target organs and susceptible animals (Gourlay and Howard 1978; Ross 1985).

Studies to determine the prevalence of *Mycoplasma* in the respiratory tract are therefore important to enable a precise definition of the distribution of *M. bovis*. This could allow the impact of the disease to be evaluated and appropriate control strategies to be designed. The purpose of this study was to investigate the distribution of *Mycoplasma* infection in the respiratory tract of slaughter cattle in north Queensland.

4.2 Materials and Methods

4.2.1 Preparation of *Mycoplasma* media

4.2.1.1 Yeast extract

Yeast extract was prepared following the method of Herderschee (1963). One kilogram of fresh bakers yeast was dissolved in 1 litre of distilled water in a five-litre flask. The mixture was heated to 80°C in a water bath with constant agitation. The pH of the mixture was adjusted to 4.5 by adding 5 N HCL and incubated at 80°C for 20 minutes. The mixture was cooled to 4°C and then the pH of the mixture was adjusted to 8.0 with 5 N NaOH. The mixture was centrifuged at 12000 *g* (Suprafuge 22 HFA, 22.50) for 40 minutes. The supernatant was then removed and filter sterilised using a 0.22 µm filter. The filtrate was dispensed aseptically into universal bottles in 20 mL amounts and stored at -20°C.

4.2.1.2 Treatment of serum

Sera from horse, cattle, pigs and humans were evaluated for use for the isolation of *Mycoplasma* from clinical samples. Before adding serum to the medium, it was tested by indirect ELISA for the presence of antibodies to *Mycoplasma* (Chapter 3). If the serum was found positive by indirect ELISA, it was acid treated to remove immunoglubulins.

In this study, pig serum was used for the propagation and isolation of *Mycoplasma*. Pig blood was collected from the abattoir and immediately transported to the laboratory. The blood was held at 4°C for six hours. The blood clots were cut using a sterile knife and the serum was separated by centrifugation at 4000 *g* (Suprafuge 22 HFA, 22.50) for 30 minutes at room temperature. After incubating the serum at 56°C for 30 minutes, the pH was adjusted to 4.3-4.5 and then held for 24 hours at 4°C. The serum was centrifuged at 12000 *g* (Suprafuge 22 HFA, 22.50) for 30 minutes. The supernatant was removed and the pH was adjusted to 7.0 with 1 N NaOH. The serum was centrifuged again at 12000 *g* (Suprafuge 22 HFA, 22.50) for 30 minutes and then filter sterilised using a 0.22 µm filter. The sterile serum was dispensed into universal bottles in 20 mL volumes and stored at -20°C. The acid treated pig serum was tested again for antibody to *Mycoplasma* by indirect ELISA.

4.2.1.3 Mycoplasma medium

Initially, the commercial Oxoid *Mycoplasma* media with a pre-prepared supplement (*Mycoplasma* supplements - G Oxoid) was used for culture of *Mycoplasma*. *Mycoplasma* broth medium was prepared by rehydrating 12.75 g of broth base in 500 mL of distilled water containing 2.5 mL of 0.4% phenol red (Sigma Chemical Co., St. Louis, MO, USA) and 1.25 mL of 10% thallium acetate (Sigma Chemical Co.). The medium was adjusted to pH 7.8, autoclaved at 121 °C at 15 kPa pressure for 15 minutes. The broth base was dispensed in 80 mL volumes and stored at -20 °C. The following filter sterilised solutions were added to each 80 mL of broth base:

- 20 mL inactivated porcine serum,
- 1 mL of yeast extract solution,
- 1 mL of 0.2% DNA solution in sodium salt isolated from salmon testes (Sigma Chemical Co.),
- 1 mL of 10% glucose solution,
- 1 mL of 1% nicotinamide adenine dinucleotide (NAD; Sigma Chemical Co.) solution,
- 1 mL of 10 mg/mL of ampicillin solution (Appendix 3.6.1).

To prepare MA, technical agar (Oxoid) was added to the broth base without phenol red and glucose to a final concentration of 1% and autoclaved as previously described. Once the temperature of the medium had cooled to about 40 °C in a water bath, the following filter sterilised solutions, were added to each 80 mL of MA base, mixed well and dispensed into Petri dishes (5 cm diameter).

- 20 mL inactivated porcine serum,
- 1 mL of yeast extract solution,
- 1 mL of 0.2 % salmon testes DNA solution in sodium salt,
- 1 mL of 10 mg/mL of ampicillin solution (Appendix 3.6.1)
- 1 mL of 1% NAD (Sigma Chemical Co.) solution

For the isolation of arginine-requiring *Mycoplasma,* modified Frey's media (Frey et al. 1968) was used. To prepare this medium, 1% arginine hydrochloride was added to modified Frey's medium (Section 4.2.1.3) and adjusted to pH 7.0. A shift of pH which caused the phenol red indicator to turn red indicated growth of *Mycoplasma* in the medium.

The efficacy of both the media for cultivating *Mycoplasma* species was first evaluated by culturing *M. bovis* and *M. arginini*.

4.2.2 Collection of specimens

A total of 500 sets of lungs from apparently normal cattle was examined at slaughter at the Townsville municipal abattoir from mid-1993 to mid-1994. Most of the cattle were female and were around 2-3 years old. Of the 500 sets of lungs examined, 80 were selected for microbiological and histopathological studies. Forty had pneumonic lesions and 40 were randomly selected as normal. Areas of hepatisation and atelectasia in the anterior lobes were regarded as typical for *M. bovis* infection (Figure 4.1). The samples were placed in individual plastic bags immediately after collection and shipped to the laboratory on ice.

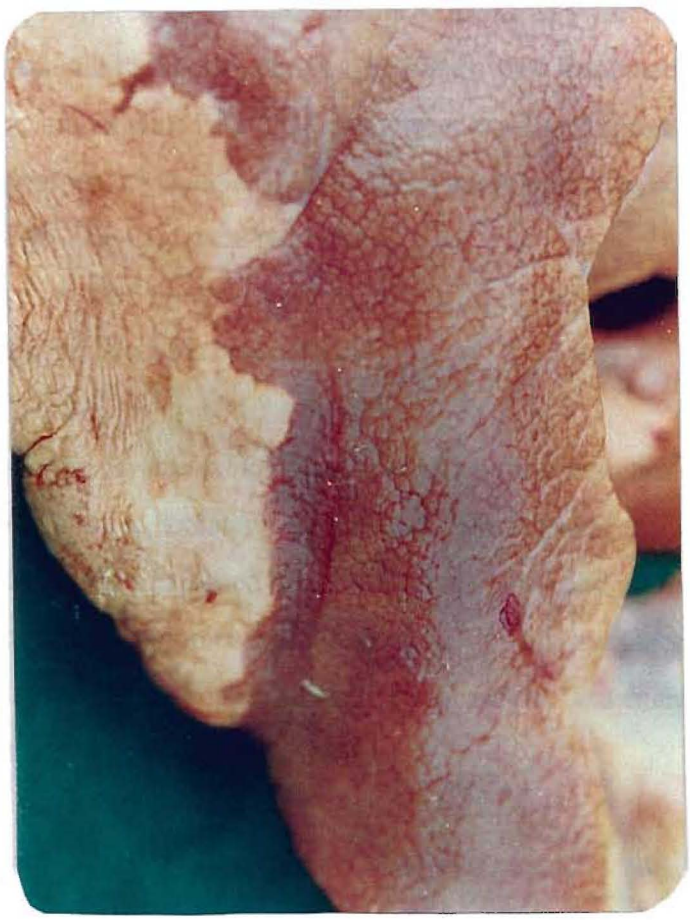

Figure 4.1 Typical local hepatisation and atelectasis in the anterior lobe of lungs obtained from cattle at the Townsville municipal abattoir.

For histopathological studies, lung tissue pieces of approximately 2-3 g from both normal and pneumonic lungs were excised and stored in 10% neutral buffered formalin.

4.2.3 Culture procedure

Approximately 1 g of lung sample was cut into small pieces with sterile scissors and homogenised using a Colworth stomacher (Townson and Mercer, Australia) in a sterile stomacher bag containing 3 mL of *Mycoplasma* medium (MM). Once a homogenous suspension was produced, it was further diluted in MM. This final suspension was a 4-fold dilution of the original lung. Samples of 300 µL were used to prepare serial 10-fold dilutions (to 10^{-4}) in Frey's *Mycoplasma* broth (FMB) and Frey's arginine broth (FAB). The tubes were incubated at 37°C in rolling drums in which the tubes rotated once per minute. The bottles were examined daily for pH (colour) changes. Once the colour of the medium turned to yellow, 300 µL of the culture was transferred to a fresh tube containing 3 mL of fresh FMB. After six to seven days, 50 µL of the contents of the tubes with the highest dilution indicating growth were spotted into the MA plates. The plates were incubated at 37°C for 14 days in a humidified atmosphere with 10% CO_2. They were examined for colonies using an inverted microscope (Olympus CK2, Japan). Differences in growth of *Mycoplasma* on plates incubated aerobically and in 10% CO_2 were recorded.

4.2.4 Staining *Mycoplasma* colonies

To better observe the *Mycoplasma* colonies to allow differentiation between *Mycoplasma* and bacterial L-form colonies, isolated *Mycoplasma* colonies were stained with Dienes stain. A light film of Dienes stain is placed on a microscopic slide and allowed to dry. A block of agar containing *Mycoplasma* colonies was cut from MA plates and placed colony-side upwards on the dry Dienes stained microscopic slide. The preparation was examined under the low-power of an inverted microscope (Olympus CK2).

4.2.5 Purification of *Mycoplasma*

Isolated *Mycoplasma* were purified by the single colony technique (Stalheim 1984). A single colony was removed by cutting out a small block of agar around the colony using a sterile scalpel. The agar block was transferred to a tube containing 3 mL of FMB and incubated for

48 hours. The culture was passed through a 0.45 μm filter, diluted 1/10 and 1/100 in FMB and then 50 μL of each dilution was spotted onto FMA and incubated at 37°C in a humidified atmosphere with 10% CO_2 for seven days. This purification procedure was repeated three times.

4.2.6 Biochemical tests

All plates and broth preparations for biochemical tests were incubated at 37°C in a humidified atmosphere with 10% CO_2 for seven days unless otherwise stated.

4.2.6.1 Glucose fermentation

One percent glucose was added to MBM containing 0.002% phenol red and adjusted to pH 7.8, then inoculated with 0.5 mL of culture medium containing the *Mycoplasma* isolate and incubated. Basal medium without glucose was also inoculated as a growth and pH indicator control.

4.2.6.2 Arginine utilisation

One percent arginine hydrochloride was added to MBM containing 0.002% phenol red and adjusted to pH 7.0, then inoculated with 0.5 mL of culture medium containing the *Mycoplasma* isolate. Basal medium without arginine was also inoculated as a growth and pH indicator control.

4.2.6.3 Haemolysis of red blood cells

Haemolytic activity of each *Mycoplasma* isolate was tested on *Mycoplasma* blood agar (MBA). Sterile blood was collected from the jugular vein of a sheep into a sterile flask containing 0.2% sodium citrate. The blood was centrifuged at 4000 *g* for 30 minutes and the supernatant removed. The red blood cells were washed three times with equal volumes of sterile PBS (pH 7.0) (Appendix 1.4). The sterile red blood cells were added to cooled MA base to a final concentration of 5%, mixed and dispensed into petri dishes (5 cm diameter) and stored at 4°C until used.

4.2.6.4 Sterol requirement

This test was carried out to discriminate the families *Mycoplasmataceae* (sterol dependant) from *Acholeplasmataceae* (sterol independent). The *Mycoplasma* isolates were cultured in FMM without serum.

4.2.6.5 Digitonin test

This test was carried out to discriminate *Mycoplasma* isolates from *Acholeplasma*, as *Mycoplasma* are more sensitive to lysis and to growth inhibition by digitonin than *Acholplasma* (Razin and Shafer 1969).

Digitonin is a steroid glycoside that interacts with cholesterol and other 3 β-hydroxy sterols to increase membrane permeability (Fiskum et al. 1980). The digitonin discs were prepared by dissolving 0.15 g of digitonin (Sigma Chemical Co.) in 10 mL of pure ethanol over a boiling water bath. Six millimetre Whatman filter discs were soaked in 0.025 mL of digitonin solution and then air dried. A MA plate was flooded with 0.5 mL of *Mycoplasma* isolate cultured in FMB and the plate was allowed to dry in a biological laminar flow cabinet. The digitonin disc was placed on the agar surface and the plate was incubated.

4.2.6.6 Phosphatase test

One millilitre of 1% (w/v) sodium phenolphthalein diphosphate solution was added to MB without phenol red and glucose and incubated. Uninoculated medium was also incubated as a negative control. After 3-4 days incubation, all media were tested by adding 5 N NaOH to a final concentration of 5%. A positive reaction was determined by a pink colour change.

4.3 Histopathology

Histopathology studies of lung tissues were carried out on 15 normal and 15 pneumonic lungs. Representative samples of formalin fixed tissues were embedded in paraffin wax and 6 μm sections were cut. The sections were stained with haematoxylin and eosin.

4.4 Results

4.4.1 Media development

Attempts to culture *M. bovis* and *M. arginini* using commercial Oxoid media were unsuccessful. As a result the modified Frey's MM was developed. For preparation of *Mycoplasma* media, sera from different animals were tested. All the sera tested had antibody titres to *M. bovis* and *M. arginini* in an indirect ELISA (Chapter 3). Culture of *Mycoplasma* species in native serum was unsuccessful. Following acid treatment, the pig serum was found satisfactory for growing *Mycoplasma* and was subsequently used in media preparation.

4.4.2 Culture of clinical samples

Some cultures were overgrown by bacterial contamination. *Mycoplasma* spp. were recovered from three (7.5%) of the 40 pneumonic lungs and from none of the normal lungs. The *Mycoplasma* spp. isolated slightly decreased the pH of MBM, and when inoculated on MA formed typical "fried egg" colonies (Figure 4.2). The Dienes stained *Mycoplasma* colonies are shown in Figure 4.3.

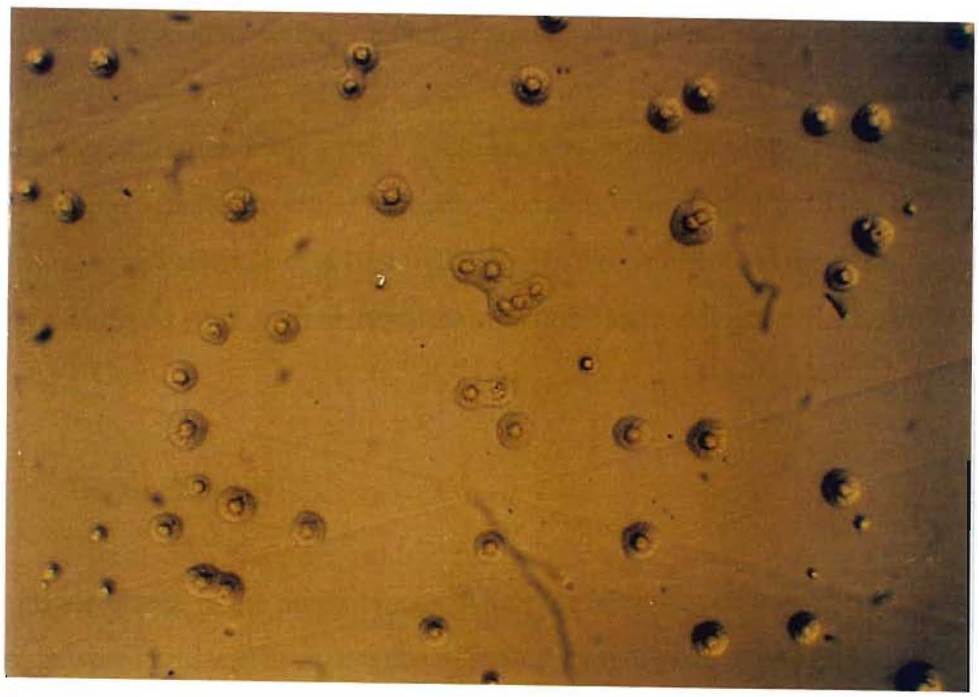

Figure 4.2 Typical "fried egg" colonies of *Mycoplasma* isolated from cattle lungs with interstitial pneumonia in the anterior lobes.

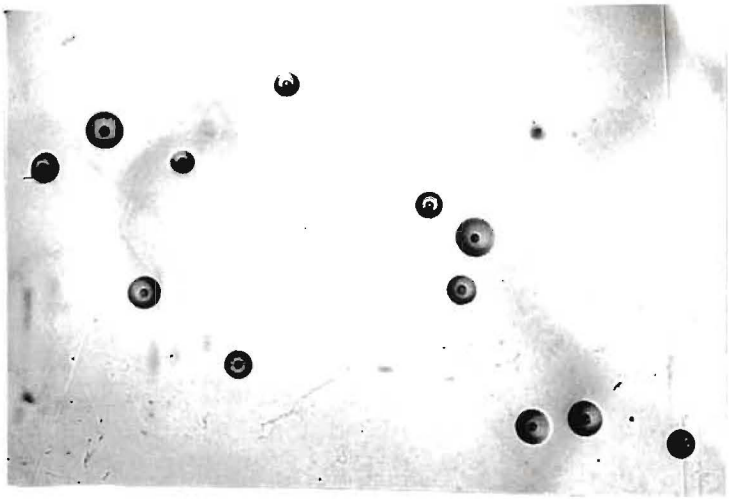

Figure 4.3 Dienes stained *Mycoplasma* colonies grown on *Mycoplasma* agar. The mycoplasmas were isolated from a pneumonic lung.

4.4.3 Biochemical tests

To discriminate *Mycoplasma* from *Acholeplasma*, the digitonin test was carried out. The zone of inhibition of the *Mycoplasma* tested was 20-30 mm. The zone of inhibition increased with increasing concentration of the stock culture and decreased with increasing incubation time. The colonies were unable to either ferment glucose or utilise arginine. They did not grow at 21°-25°C, or in the absence of serum. All colonies were positive in the phosphatase test and zones of haemolysis were seen on mycoplasma blood agar. All isolates required 10% CO_2 for growth at 37°C.

4.4.4 Histopathology

No lesions were seen in the normal lungs. All of the lesions seen in lungs showing signs of pneumonia were similar. They were characterised by moderate and focal thickening of the inter-alveolar septa by a mostly mononuclear cell proliferation. There was also hyperplasia of bronchiolar epithelium and atelectasis in some locations. Moderate "cuffs" or "sheets" of

lymphocytic hyperplasia surrounded some affected bronchioles. Cuffing interstitial pneumonia was also recognised (Figure 4.4).

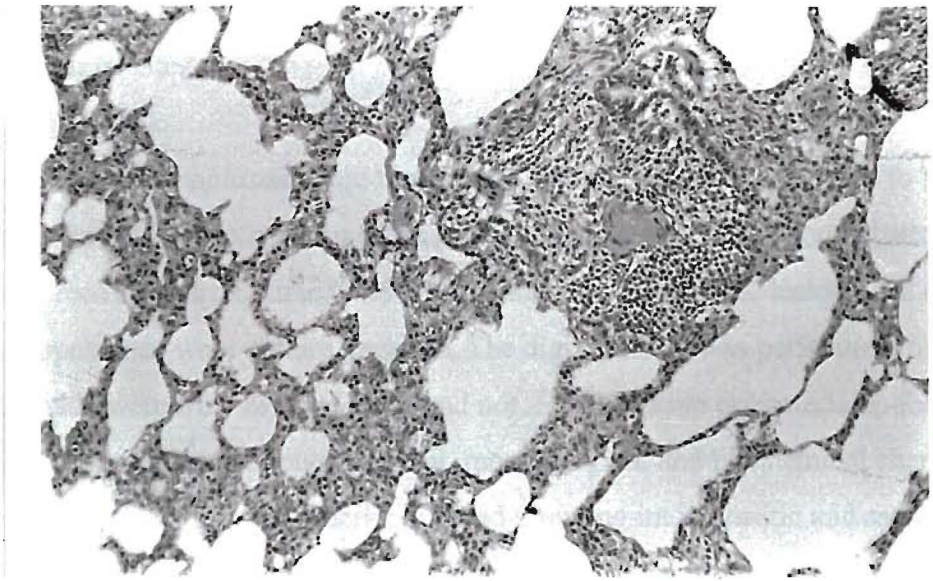

Figure 4.4 Microscopic lesions of lungs associated with *Mycoplasma bovis* showing lymphocytic infiltration and thickening of the interstitium (interstitial pneumonia). Stained with haematoxylin and eosin (H&E) (Magnification x226.8).

4.5 Discussion

The general principal in cultivating *Mycoplasma* is to prepare an environment to culture the organism resembling as closely as possible the ecological niche in the host (Simecka et al. 1992). The fact that these organisms have the smallest genome among prokaryotes explains their lack of many biosynthetic pathways and as a result, their marked dependence on the stock of many nutrients supplied by their host or by artificial culture media. Unfortunately, in many cases, imitating the natural elements provided by the host is very difficult *in vitro*. As a result many *Mycoplasma* may fail to grow (Razin 1994).

Current methods for the diagnosis of animal *Mycoplasma* infections are generally based on cultivation of organisms from clinical samples followed by serological identification of the isolate (Kenny 1992; Simecka et al. 1992). Culture takes time and employs relatively costly media. Because of common antigens and cross reactivity between *Mycoplasma* species, the serum for preparation of *Mycoplasma* media should be from *Mycoplasma* free animals (Jasper

1981; Stalheim 1984). In this study a number of the sera from different animals (pigs, horses, cattle and human) had antibodies against *M. bovis* and *M. arginini* and this may explain why the type strains failed to grow in *Mycoplasma* media which contained untreated native serum. *Mycoplasma* media containing acid treated pig serum was found the best for growing *Mycoplasma* isolates.

The isolation of *Mycoplasma* from the lung lesions of cattle was attempted to try to determine the prevalence of *Mycoplasma* in cattle processed in a north Queensland abattoir. *Mycoplasma* spp. were recovered from three (7.5%) lungs showing pneumonic lesions. All of the lungs of normal appearance were culture negative. The digitonin test was performed to ensure that the isolates made were truly *Mycoplasma*, and not *Acholeplasma* or bacterial L-forms capable of growing on the media. Although cultural, morphological and biochemical characteristics of *Mycoplasma* are not species-specific, a broad grouping into parasitic and saprophytic organisms is recognised based on sugar fermentation and serum requirements (Freundt 1958). The *Mycoplasma* isolates were tentatively characterised as *M. bovis* based on failure to ferment glucose and hydrolysis arginine, haemolysis sheep red blood cells and positive phosphatase activity. The identity of the isolates was subsequently confirmed by an indirect ELISA using the MAbs prepared from a reference strain (Chapter 5) and by PCR (Chapter 8).

The microscopic changes seen in affected tissue, mild interstitial pneumonia, cellular infiltration and lymphocytic hyperplasia surrounding affected bronchioles, have been reported as characteristic for various *Mycoplasma* respiratory infections (Bryson et al. 1978; Gourlay and Howard 1979, 1982; Howard et al. 1987). Moreover these changes have been induced in gnotobiotic calves by intratracheal injection of *M. bovis* alone (Thomas et al. 1986). However, the presence of these changes alone is not pathognomic for the disease. Establishing a diagnosis based solely on the presence of either gross or microscopic lesions may lead to false negative results, and the diagnosis may be influenced by the number of lungs examined from the herd in question and the resolution of the lesions by the time of examination (Simecka et al. 1992). Although all of the lesions observed in pathological sections were similar, *M. bovis* was isolated only from three of the lesions. However, these results suggest that *Mycoplasma* is present in pneumonic lesions in cattle in north Queensland. The low number of *Mycoplasma* isolated may reflect the low sensitivity of culture, compared with histopathology or other methods described in subsequent chapters.

Isolation of *Mycoplasma* in this study may have been thwarted by low numbers of *Mycoplasma* in the samples, through contamination with other bacteria, or a deficiency of some nutrients following acid treatment of the serum. It has been reported that culture is an insensitive screening method for detection of *Mycoplasma* infections in the respiratory tract (Thomas et al. 1987b) and that for *in vitro* isolation of *Mycoplasma* at least 10^2 to 10^3 CFU *M. bovis* are required (Razin 1994).

It has been reported that the currently available commercial media do not support the growth of pathogenic *Mycoplasma* (Razin 1994). Insufficient nutrients in the *Mycoplasma* media (Simecka et al. 1992) or the presence of *Mycoplasma* inhibitors in the clinical specimens (Kirchhoff et al. 1982) could also be responsible for the failure to grow the organisms. However, the isolation of *Mycoplasma* from clinical specimens is important to enable detailed studies on those pathogens. Thus, further work is required on a more sensitive screening method to enable the prevalence of *M. bovis* and its association with disease in cattle to be evaluated.

CHAPTER 5

PRODUCTION OF A MONOCLONAL ANTIBODY WITH A UNIQUE SPECIFICITY FOR *MYCOPLASMA BOVIS*

5.1 Introduction

Conventional methods for the diagnosis of infectious disease are quickly being substituted by techniques that include the use of MAbs (Hall 1988). Hybridoma technology is also being applied to *Mycoplasma* research to increase the understanding of antigenic variation, virulence, and the host immune response (Olson et al. 1991; Feld et al. 1992; Panangala et al. 1992).

Diagnostic methods employing MAbs are more specific than the classical methods such as indirect haemagglutination (IHA), the CFT and indirect ELISA (Jones 1989a). Thus, MAbs may provide useful immunochemical tools for the identification of *Mycoplasma* species and for overcoming the problems of cross-reaction between closely related *Mycoplasma* species.

The aim of this study was to develop a highly specific MAb to *M. bovis*, that could be used in a serological test to discriminate *M. bovis* from other bovine *Mycoplasma* infections. In this chapter, the production of a MAb with unique specificity to *M. bovis* is described.

5.2 Materials and Methods

5.2.1 *Mycoplasma* strains

The *Mycoplasma* strains used in this study are listed in Table 5.1. Most of the *Mycoplasma* strains were obtained from the National Collection of Type Cultures (Colindale, UK) and some strains were obtained from the Queensland Department of Primary Industry's Oonoonba Veterinary Laboratory at Townsville, north Queensland.

Table 5.1 Strains of *Mycoplasma* used in the produce of monoclonal antibody production.

Species	Strain
Mycoplasma bovis	NC10131[a]
Mycoplasma bovis	PG-45 strain[b]
Mycoplasma ovipneumoniae	NC10151[a]
Mycoplasma agalactiae	NC10123[a]
Mycoplasma bovigenitalium	NC10122[a]
Mycoplasma bovirhinis	NC10118[a]
Mycoplasma dispar	NC101125a
Mycoplasma group 7	Leach N29[b]
Mycoplasma arginini	WCa 7880[b]

[a] Type strain NCTC, National Collection of Type Culture
[b] Oonoonba Veterinary Laboratory, Townsville, north Queensland

5.2.2 *Mycoplasma* culture

A single colony of each *Mycoplasma* strain was selected on MA, inoculated into 250 mL of MM (Chapter 4) in a 1 litre flask and incubated with constant agitation at 37°C. The *Mycoplasma* were harvested and purified as described previously (Section 3.2.2). Aliquots were stored at -70°C.

5.2.2.1 Titration of viable Mycoplasma

The number of viable cells in the MB culture was determined by calculating the number of CFU in a titration experiment. The MB culture was diluted 10-fold and aliquots were cultured on the surface of MA and then the number of colonies was counted and the CFU present in the broth calculated (Rodwell and Whitecomb 1983).

5.2.3 Production of monoclonal antibodies

5.2.3.1 Immunisation schedule

Ten 6-week-old BALB/c mice were immunised intraperitoneally (IP) with *M. bovis* whole cell antigens in PBS (10^9 cells/mL). To prepare the whole cell antigens the cells were lysed as described in Section 3.2.3.1. The sonicated cells were emulsified with an equal volume of Freund's complete adjuvant (Gibco, USA; Cat.No:15721-012) by repeatedly aspirating the mixture using a 25-gauge needle and 2 mL syringe. The resultant inoculum was thick and milky. For the first immunisation, the mice were injected IP with 200 µL of the emulsion. Two weeks later, the mice were boosted using a cell sonicate in Freund's incomplete adjuvant (Gibco; Cat. No:15720-014) prepared as described for the first immunisation. At least two further injections without adjuvant were given at two week intervals. The mice were tail bled and the sera tested for antibody using an indirect ELISA. Three days before fusion, the mice were further boosted intravenously with 200 µL of sonicated *M. bovis* without adjuvant.

5.2.3.2 Preparation of spleen cells

The immunised mice were killed using carbon dioxide gas. The dead mice were fixed on a board and blood was collected from the axillary plexus of the left-front leg or directly from the heart. The spleen was aseptically removed and placed in a 20-mL sterile universal bottle containing 10 mL of CSL medium (Appendix 2.2), prewarmed to 37°C (without serum added) then transferred to a petri dish. The spleen was then transferred to a Petri dish, trimmed and then teased apart using a 19-gauge needle in a 5.0 mL syringe. The cells were released from the spleen by repeated aspiration of the cell suspension. Any remaining clumps of cells were disrupted by using two bent 25 gauge-needles. The cell suspension was transferred to a 50-mL conical centrifuge tube and diluted to 20 mL with CSL medium.

5.2.3.3 Cell count and calculation of cells

To determine viability the spleen cells were stained by Trypan blue exclusion. Briefly, a small aliquot of spleen cells was mixed with an equal volume of 0.14% Trypan blue (Appendix 2.1) and the non-staining cells were counted using in a haemocytometer. The total number of viable lymphocytes was calculated as follows:

Total number of stained cells x 10^4 = Number of viable cells/mL

Number of cells/mL x volume of cell suspension (20) = Total number of spleen cells

As approximately half of the cells present in a spleen cell suspension are considered lymphocytes, the total number of spleen cells was divided by two, to give an estimate of the number of lymphocytes.

5.2.4 Preparation of the myeloma cells

One week before fusion a frozen ampoule of SP2/O myeloma cells was removed from storage in liquid nitrogen, and held in a container of dry ice before being placed in a water bath at 37°C. When the suspension thawed the vial was wiped with 70% ethanol and the contents of the ampoule diluted with 9 mL of prewarmed CSL medium (Appendix 2.2) containing 20% new born calf serum (NBCS) (Appendix 2.3). The suspension was centrifuged at 35 *g* (Clements, Australia; 2000) for 5 minutes. The supernatant was then discarded and the cell pellet was resuspended in 10 mL of fresh culture medium, transferred to a small culture flask and incubated at 37°C with the cap loosened in a humidified atmosphere of 5% CO_2.

The myeloma cells were maintained by a one in four split and addition of fresh culture media every 3-4 days. Two or three days before the fusion, the cells were split to provide a monolayer of actively dividing cells that were approximately 80% confluent. The number of myeloma cells was determined using a Trypan blue count with a haemocytometer (as described in Section 5.2.2.3). The appropriate volume of media containing myeloma cells was centrifuged at 35 *g* (Clements) for five minutes at room temperature and the supernatant returned to the original flask and placed in the incubator.

5.2.5 Fusion procedure

To generate MAbs, spleen cells were immortalised by fusion with myeloma cells. This procedure was carried out in a 37°C water bath and all manipulations were performed in a biological laminar flow cabinet. The fusion media consisted of 1 g of solid polyethylene glycol (PEG) 4000 (Koch-Light, England; Cat. No. 4807-00) autoclaved at 121°C for 15 minutes and, while molten, 1.0 mL of CSL medium (without serum) was added to give a final

concentration of 50% PEG. This media was maintained at 37°C to keep it liquid until required. The myeloma cells were added to the spleen cells in the proportion 1:10 and the suspension gently mixed. The resulting cell suspension was centrifuged at 35 *g* for five minutes at room temperature and the supernatant discarded. The pellet was gently loosened by tapping the tube and then, at one minute intervals, the 50% PEG was added drop-wise while gently rotating the tube. The cells were left to stand for one minute in order for the fusion to take place. One millilitre of CSL medium was added slowly within one minute, and within the next five minutes a further 20 mL CSL medium was added at a rate of 4 mL per minute. The fused cell suspension was centrifuged at 35 *g* (Clements) for five minutes at room temperature. The supernatant was discarded and the pellet resuspended very gently in 50 mL of myeloma cell conditioned culture media and returned to the 80-cm^2 tissue culture flask in which the myeloma cells had grown. The fused cells were then incubated overnight at 37°C with the cap loosened in a humidified 5% CO_2 atmosphere.

The following day the attached cells were gently washed off the flask with a sterile Pasteur pipette, and the cell fusion mixture made up to 100 mL with CSL medium supplemented with 20% NBCS and 1 mL solution hypoxanthine aminoptrin thymidine (HAT) (Appendix 2.4). The cell suspension was then dispensed into ten 96 well flat-bottomed tissue culture plates (100 µL/well) (NUNC, Denmark; Cat. No. 1 67008). Because hybrids occur in these mixtures at very low frequency, the parental cell lines need to be removed to allow the detection of the hybrids. HAT medium contains aminopterin that blocks one of the major metabolic pathways involved in DNA synthesis. Hence cells in this selective medium must use an alternative pathway to synthesise DNA and survive. Two enzymes, thymidine kinase (TK) and hypoxanthine phosphoribosyl transferase (HPRT) and their substrate thymidine and hypoxanthine are required to permit this alternative pathway to be used. The parental myeloma cells are deficient in HPRT and thus cannot grow in HAT medium. Spleen cell lymphocytes which cannot divide are the source of enzyme HPRT for hybridoma cells. HAT medium supplies the hypoxanthine and thymidine necessary as substrate for HPRT, and as a result only hybrid cells can grow in HAT medium (Jones 1989b).

5.2.6 Preliminary culture of hybridomas

The tissue culture plates containing hybrid cells were checked for cell proliferation after seven days. Those wells containing large round cells were marked. The hybridoma cultures were

maintained by removing 50 µL/well of supernatant and adding 100 µL/well of fresh CSL medium supplemented with 20% NBCS and 1% HAT solution. Maintenance of hybridoma cultures in this manner improves the general health of the hybridomas and will keep them rapidly growing throughout the screening procedure. The marked wells were checked periodically for further cell growth. Once the cells reached 75-80% confluency, the supernatants were screened for *M. bovis*-specific antibody using the indirect ELISA (Section 3.2.4).

5.2.7 ELISA screening of hybridoma supernatants

Supernatants from wells containing hybridomas were tested for specific MAb using the indirect ELISA described in Section 3.2.4. The working dilutions of antigen and conjugate had been previously determined (Section 3.2.4). Briefly, 96 well U bottom microtitre plates (NUNC Immunoplate) were coated with 100 µL/well of antigen diluted in 1:800 (0.2 µg/well) in coating buffer (pH 9.6) (Appendix 1.7), sealed, and incubated at room temperature overnight. The plates were washed three times with PBS-T and tapped dry.

An aliquot of 50 µL of tissue culture supernatant was removed aseptically from the surface of each well without disturbing hybridomas at the base of the well. The hybridoma cultures were topped up with fresh medium and re-incubated. The tissue culture supernatant collected was transferred to the ELISA (antigen coated) plate. Murine polyclonal *M. bovis* antisera was used as a positive control sera and mouse pre-immune serum and supernatant from tissue culture cells without hybridomas were used as negative control sera. The plate was incubated for one hour at room temperature and then washed three times with PBS-T and tapped dry. Affinity purified goat anti-mouse IgG conjugated to HRPO (Bio-Rad; Cat. No. 170-6516) was diluted 1/2000 in TEN-TC. Aliquots of 50 µL were transferred to each well and the plate incubated for one hour at room temperature. Finally the plates were washed three times with PBS-T, tapped dry and incubated with freshly prepared enzyme substrate (Appendix 1.10) for another hour. After one hour, the colour intensity was measured using a spectrophotometer (Model 3550; BioRad, California) at dual wavelengths of 415 and 490 nm. Depending on the relative success of the fusion, a cut off OD of 0.5 or 1.0 was used to determine which hybridoma lines were to be further cultured.

5.2.8 Culturing and cryopreservation of hybridomas

After positive wells were identified, the cells were washed off the plastic surface with a sterile plugged Pasteur pipette. The cells were transferred to 24 well tissue culture plates (Linbro, Flow Laboratories Inc., USA; Cat. No. 76-063-53) and, after sufficient growth had occurred, to 25 cm^2 tissue culture flasks. Once the hybridomas were transferred to the 24 well plates the HAT was replaced with hypoxanthine thymidine (HT) (Appendix 3.5). On transfer to the 25 cm^2 flask, the HT was omitted from the culture medium. When the cells in the flasks reached a confluency of at least 70%, they were washed from the plastic surface and the whole suspension centrifuged at 35 *g* (Clements) for five minutes. The cell supernatant was poured off carefully into a sterile bottle, sodium azide added to a final concentration of 0.01% and the supernatant stored at 4°C. The cell pellet was resuspended in 900 µL of CSL media. It was transferred immediately into a 1 mL plastic screw-cap cryopreservation tube (NUNC; Cat. No. 3-66656) with 100 µL dimethyl sulfoxide (DMSO). The plastic ampoule was held at -70°C overnight before transfer to storage in liquid nitrogen. When required, the frozen ampoules were removed from liquid nitrogen stocks, thawed and cultured in CSL medium containing 20% NBCS, as described in Section 5.3.2, then incubated at 37°C with the cap loosened in a humidified 5% CO_2 atmosphere.

5.3 Characterisation of Monoclonal Antibodies

5.3.1 Cloning of hybridoma lines

Cloning was carried out using the limiting dilution method. Briefly, the cells were dislodged from the plastic surface and the number of hybridoma cells in the supernatant were counted as described in Section 5.2.3.1. Based on the number of cells present, an aliquot of the cell suspension was diluted in 20 mL of culture medium to give a final concentration of 20 cells/mL. A 96 well, flat-bottom, tissue culture plate (NUNC; Cat. No. 1 6 7008) was divided into four sections. In the first section consisting of 24 wells, 200 µL of cell suspension and 50 µL of culture medium were added to each well. In the second section, 100 µL of cell suspension and 150 µL of culture medium were added to each well. In the third section, 50 µL of cell suspension and 200 µL of culture medium were added to each well and in the fourth section, 25 µL of cell suspension and 225 µL of culture medium were added to each well. Statistically, there would be four cells per well in section one and sections two, three and four

would have had two, one and 0.5 cells per well, respectively. After 3-4 days the plates were checked for growth and those with a single, discrete cell were marked. Once hybridoma colonies were of sufficient size (50% confluence) the supernatant were screened for antibody by indirect ELISA (as described in Section 3.2.4). Clones from the three wells with the fastest growing cells and with a high OD value in the indirect ELISA were selected and cultured into 24 well tissue culture plates (Linbro; Cat. No. 76-063-05). When ready to be split, the cells were re-cloned by re-culture in 24 well tissue culture plates.

Once a line had been cloned twice and was re-established, replicates were placed into storage in liquid nitrogen and the supernatant stored at 4 °C for further study as described in Section 5.2.8. The cloned hybridoma supernatants were tested by indirect ELISA (Section 3.2.4) before they were used for ascitic fluid production.

5.3.2 Affinity of monoclonal antibody

The affinity of MAbs to *M. bovis* was estimated by a modification of the indirect ELISA described in Section 3.2.4. Serial twofold dilutions were made in antigen coated ELISA plates. Hybridoma culture supernatants (undiluted) were added to the first column of the plates and twofold dilutions were made in TEN-TC across to column 12. The assay was developed as previously described and absorbance values recorded.

5.3.3 Specificity of monoclonal antibodies

Cell culture supernatants were tested by indirect ELISA (described in Section 3.2.4) for reactivity with other *Mycoplasma* species including *M. arginini, M. agalactiae, M. bovirhinis. M. group 7, M. dispar, M. ovipneumoniae, M. bovigenitalium* and *M. bovis*. Briefly, the protein concentration of each *Mycoplasma* antigen was adjusted to 0.5 mg/mL (Chapter 3). A 96-well plate (NUNC; Cat. No.167008) was coated in duplicate with 0.2 µg/well of protein from each *Mycoplasma* antigen diluted in coating buffer (pH 9.6) (Appendix 1.7). The plates were incubated overnight and then the indirect ELISA (described in Section 3.2.4) was carried out. Further assessment of specificity was carried out after purification and concentration of MAb E4 with different dilutions of *Mycoplasma* antigen.

5.3.4 Monoclonal antibody production as ascitic fluid

For the production of ascitic fluid, 10 BALB/c mice, 6-10 weeks old were injected IP with 200 µL of 2,6,10,14-tetramethylpentadecane (Pristane) (Sigma; Cat. No. T-7640). Cloned hybridomas (as described in Section 5.3.1) were pelleted by centrifuging at 35 *g* for five minutes (Clements). The cells were gently washed by resuspending in 10 mL serum free culture medium and resuspended in 500 µL of serum free culture medium. A volume of 10^7-10^8 cells per mouse was injected IP. Mice were observed for the development of ascitic fluid and when sufficiently swollen, were killed using CO_2 gas. The ascitic fluid was collected in a 10-mL centrifuge tube from an 18-gauge needle inserted into the peritoneum. The ascitic fluid was clarified by centrifugation at 1700 *g* (Suprafuge 22 HFA, 22.50) for 30 minutes to remove any clots or cell debris. Phenyl methyl sulfonyl fluoride (PMSF) was added to the supernatant at a final concentration of 1 mM and stored at -70°C. The ascitic fluid was tested for specificity in an indirect ELISA (Section 3.2.4) before it was purified or used for any further characterisation.

5.3.5 Determination of the immunoglobulin isotype

A Mouse Isotyper Panel (Bio-Rad; Cat. No. 172-2055) used with a modification of the indirect ELISA procedure (described in Section 3.2.4) was used to determine the Ig isotype of the hybridoma culture supernatant and clarified ascetic fluid. Briefly, the plate was coated with 100 µL of crude *M. bovis* antigen diluted in coating buffer at 0.2 µg/well (Appendix 1.7). After incubation overnight at room temperature, the plate was washed three times with PBS-T (Appendix 1.8), tapped dry and 50 µL/well of MAb (prepared as in Section 5.3.3 and 5.3.4) was added to the plate. The plate was incubated for one hour at room temperature then washed three times with PBS-T and tapped dry. Aliquots of 50 µL/well of affinity purified rabbit anti-mouse Ig reagent (Bio-Rad; Cat. No. 172-2055) were added to one row for each isotype (IgG_1, IgG_2, IgG $_2$B, IgG_3, IgA, IgM, anti Kappa and anti Lambda chain) and incubated for two hours at room temperature, then the plate was washed three times with PBS-T and tapped dry. Affinity purified anti-rabbit IgG-HRPO (Bio-Rad; Cat. No. 172-1013) diluted 1/1000 in TEN-TC (100 µL/well) was added to each well and the plate was incubated for one hour at room temperature, washed three times with PBS-T and tapped dry again. Finally, 100 µL/well of substrate solution (Appendix 1.10) was added to the plate and incubated for one hour at room

temperature. The absorbance was monitored on a microplate reader at the dual wavelengths of 415 and 490 nm.

5.3.6 Purification of monoclonal antibody using protein A chromatography

The MAb was purified using protein A sepharose CL-4B beads (Pharmacia, Uppsala; Cat. No. 71-7090-00-01). Briefly, one gram of protein A suspended in 200 mL distilled water was filtered through a 0.45 μm filter (Sartorious, Germany; Cat. No. 165 55 K), added to a 10 mL bench top column (BioRad) and left to settle. The column was equilibrated with three bed volumes of Tris buffer (pH 7.4). The ascitic fluid was adjusted to a pH of 8.0 by adding 1/10 volume of 1 M Tris buffer (pH 8.0), clarified by centrifugation at 10000 *g* in a microcentrifuge (Microfuge E™; Beckman, UK) and passed through a 0.45 μm filter (Sartorious; Cat. No. 165 55 K). The ascitic fluid was loaded onto the column and after washing the column with three bed volumes of Tris buffer (pH 7.4) to remove any unbound contamination, the MAb was purified with both low salt and high salt procedures as described by Harlow and Lane (1988). The bound IgG_1 fraction was eluted from the beads with three bed volumes of 100 mM glycine buffer (pH 3.0). One millilitre fractions were collected in tubes containing 300 μL of Tris buffer (pH 9.0) to neutralise the pH of the glycine buffer. The fractions were screened for activity by indirect ELISA (as described in Section 3.2.4). Fractions found to be positive in the ELISA were pooled and concentrated using micro-concentrators (Centricon 30 Amicon USA; Cat. No. 4203) and centrifugation of 1000 *g* at 4°C with an angled rotor (Superfuge 22 HFA, 22.50).

Following this procedure the fractions found to be negative by ELISA were collected separately and purified using a high salt method. The NaCl concentration of the collected fractions was adjusted to 3.3 M by freshly adding 1/10 volume of 1.0 M sodium borate (pH 8.9) and the fractions then passed through a protein A column. The procedure described by Harlow and Lane (1988) was followed, and involved running 50 mL of 3.0 M NaCl, 50 mM sodium borate (pH 8.9) though the column and then running 50 mL of 3.0 M NaCl, 10 mM sodium borate (pH 8.9) through the column to wash off any unbound material. The bound IgG_1 fraction was eluted from the beads with three bed volumes of 100 mM glycine buffer (pH 3.0) and screened for activity by indirect ELISA (as described in Section 3.2.4) and ELISA positive fractions were pooled and concentrated as described for the low salt method. The MAbs concentrated by both low salt and high salt methods were mixed.

5.3.7 Protein estimation of MAb isolated from mouse ascitic fluid

The MAbs purified by column chromatography were extensively dialysed in 1/10 × PBS at 4°C. The protein concentration of the MAb was estimated using either a spectrophotometer reading at 280 nm (1 OD = 0.8 mg/mL protein) (Harlow and Lane 1988) or the BSA protein assay kit (Pierce, Illinois, USA; Cat. No. 23 225). Briefly, in a series of six glass tubes, 0, 5, 10, 20, 30 and 40 µL of 2 mg/mL BSA was mixed with 100, 95, 90, 80, 70 and 60 µL of distilled water and used in the construction of a standard curve. Samples of unknown protein concentration were set up as follow: no dilution, 1:5 and 1:10 dilutions in distilled water such that the final volume was 100 µL. The working solution of reagents A and B provided with the kit was made by addition of 1 mL reagent B (4% $CuSO_4$, 5 H_2O) to 49 mL of reagent A (sodium carbonate, sodium bicarbonate BCA detection reagent and sodium tartrate in 0.1 N NaOH). A volume of 2 mL of working solution was added to each of the standard and unknown concentration samples, mixed well and the mixture incubated at 37°C for 30 minutes. Duplicate 250 µL aliquots of each standard and unknown concentration sample were transferred into wells in a 96-well ELISA plate and the absorbance was read using a microplate reader at the dual wavelength of 540 and 562 nm (Bio-Rad; Model 3550). Using the absorbances from the standard solutions of known protein concentration, the protein concentration of the unknown samples could be determined.

5.3.8 Conjugation of monoclonal antibody to horse radish peroxidase

The MAb E4 prepared as in Section 5.3.3 was concentrated to 8 mg/mL using a microconcentrator (Centricon 30 Amicon; Cat. No. 4208) as measured by the BSA method (Section 5.3.7). The conjugation procedure with the MAb and HRPO was as follows.

Briefly, HRPO granules (Boeheringer Mannheim, Germany; Cat. No. 814 407) were dissolved in distilled water (4 mg/mL) to give a final ratio of 1 mg HRPO for every 2 mg of antibody. Fifty microlitres of freshly prepared 0.1 M sodium periodate was added per millilitre with gentle stirring for 20 minutes at room temperature, by which time the HRPO was activated and the solution had become green in colour. This solution was passed through a Sephadex G25 column (Pharmacia; Cat. No. 17-0032-01) which had been pre-equilbrated with 1 mM sodium acetate buffer (pH 4.4) and 300 µL fractions were collected. The orange coloured fractions were pooled and added to the MAb 8 mg/mL, which had 200 µL of sodium

carbonate buffer (pH 9.5/mL). The solution was gently mixed for two hours at room temperature and dialysed extensively at 4 °C in PBS. The conjugated antibody was aliquoted in 200 µL volumes and stored at 4 °C. The MAb E4 conjugated to HRPO was tested by indirect ELISA (Section 5.2.7) and compared with unconjugated MAb for activity.

5.3.9 Determination of specificity of monoclonal antibody E4 by Western blot

Whole cell lysates of various mycoplasma species were resolved, and transferred to a nitrocellulose membrane according to the methods of SDS-PAGE.

5.3.9.1 SDS-PAGE

SDS-PAGE was carried out following the procedure described by Laemmli (1970) with minor modifications. Briefly, electrophoresis in a 12% polyacrylamide gel was performed using a Mini Protean II system (BioRad). The glass plates were cleaned first with soap and distilled water, dried, and wiped thoroughly with acetone and lint-free tissues. The plates were clamped together in the gel casting apparatus using 1.0 mm spacers. Polyacrylamide gel was prepared using a 30% (w/v) bis-acrylamide stock solution. To prepare a 12% gel 4.8 mL of bis-acrylamide stock solution, 3 mL of 1.5 M Tris-HCl buffer (pH 8.8) (Appendix 2.7), 4.2 mL distilled water and 40 µL of freshly prepared 10% (w/v) ammonium persulphate (APS) were mixed in a conical flask and degassed for five minutes by slowly bubbling through nitrogen gas at 4 ° C. Polymerisation was initiated by adding 10 µL N,N,N,N,N, tetramethylenediamine (TEMED).

The gel solution was mixed gently, poured into the space between the glass plates and overlaid with a thin layer of butanol until completely polymerised. The acrylamide was judged to be polymerised when an additional line at the interface with the butanol was observed near the top of the gel. The butanol and unpolymerised acrylamide were then removed from the top of the separating gel by rinsing several times with distilled water and drying with a paper tissue.

A 4.5% stacking gel was prepared by mixing 0.75 mL bis-acrylamide stock solution, 1.25 mL 0.5 M Tris-HCl buffer (pH 6.8) (Appendix 2.8), 3 mL of distilled water and 20 µL of freshly prepared 10% (w/v) APS in a conical flask, and the solution was degassed. After the addition

of 10 µL of TEMED, the stacking gel was poured on top of the separating gel. A 10-well 0.5 cm comb was inserted at an angle taking care not to trap any air bubbles. The solution was allowed to polymerise until interface lines were seen around the comb. Following polymerisation, the comb was removed and gels were clipped onto the electrode unit of the Mini Protean II apparatus. The electrode was set up in a buffer tank filled with 1 × electrode buffer (Appendix 2.10)

5.3.9.2 Preparation of Mycoplasma proteins

A 10-mL volume of mid logarithmic phase culture of each of *M. bovis, M. arginini, M. bovigenitalium, M. dispar, M. ovipneumoniae, M. agalactiae, M. bovirhinis* and *M.* group 7 (Section 5.2.2) was centrifuged at 10000 *g* (Suprafuge 22 HFA, 22.50) for five minutes. The supernatant was discarded and the pellet was washed twice with 1 mL of PBS (pH 7.0) (Appendix 1.4), transferred to a microfuge tube, centrifuged at 10000 *g* (Microfuge E™) for three minutes and the supernatant discarded. The pellet was resuspended in 150 µL distilled water and 50 µL of reducing buffer was added (Appendix 2.9). The samples were held in a waterbath at 100°C for five minutes to denature any proteins, then centrifuged at 10000 g (Microfuge E™; Beckman, UK) at 4°C for 20 minutes to remove cell debris. The supernatant was transferred to a new microfuge tube, labelled and stored at -20°C.

Aliquots of 10 µL of each *Mycoplasma* and SDS-PAGE pre-stained protein standards (Bio-Rad; Cat. No. 161-0318) were loaded into separate wells. A constant potential difference of 200 volts was applied across the gels until the tracking dye reached the bottom of the gel.

To visualise the protein profile of the *Mycoplasma* species tested the gel was carefully separated from the glass plates and immersed in a mixture of fixative and Coomassie Blue stain (Appendix 2.11) for two hours. The gels were destained overnight using several changes of destain solution (Appendix 2.12).

5.3.9.3 Western blotting

After electrophoresis, the polypeptides were transferred to a nitrocellulose membrane (Bio-Rad) using a Mini Trans-Blot apparatus (Bio-Rad; Cat. No. 170-3930). Briefly, the gel holder was placed in a shallow container filled with transfer buffer (Appendix 2.13) with a cathode

panel (grey in colour) flat on the bottom. A pre-soaked fibre pad was placed over the cathode panel, followed by a piece of saturated filter paper and the gel. The gel was aligned and covered with pre-wetted nitrocellulose membrane (Bio-Rad Transblot, 0.45 μ, Cat. No. 162-0115). A glass tube was rolled over the membrane to remove any trapped air bubbles. The nitrocellulose membrane was covered with pre-wetted filter paper and a fibre pad. The gel holding cassette was closed and then placed in the buffer tank filled with transfer buffer such that the cathode of the gel holding cassette connected with the appropriate electrode. The proteins were transferred to the nitrocellulose membrane at 30 volts overnight at 4 °C.

The nitrocellulose membrane was removed from the apparatus and successful transfer was confirmed by observation of transfer of pre-stained markers. The membrane was washed by immersing in PBS-T (Appendix 1.8) on a rocker. The membrane was washed 3 times for 20 minutes each in fresh PBS-T at room temperature. The nitrocellulose membranes were blocked in 5% skim milk in PBS-T for one hour and washed 3 times for 10 minutes in fresh PBS-T at room temperature. The nitrocellulose was immersed in MAb E4 diluted 1/8000 in TEN-TC (Appendix 1.9) and rocked gently for one hour at room temperature. After washing with PBS-T for 3 times for 20 minutes, the nitrocellulose was exposed to goat anti-mouse HRPO (Bio-Rad Cat. No.172-1011) at 1/1500 dilution in TEN-TC (as secondary antibody) for one hour at room temperature. The nitrocellulose was washed again 3 times for 20 minutes each with PBS-T and blots were stained with 3,3 Diaminobenzidine tetrahydrochloride dihydrate (DAB) (Sigma; d-5637) substrate solution containing 30 mg DAB in 100 mL PBS (pH 7.0) (Appendix 1.4) and 30 μL of 30% stock H_2O_2 for one to five minutes or until the desired colour intensity had developed. The nitrocellulose was washed with several changes of distilled water, air dried and stored in a sealed plastic bag.

5.3.10 Sensitivity of the monoclonal antibody

To test the sensitivity of MAb E4, *M. bovis* antigen was adjusted to 10 μg/mL in coating buffer (Appendix 1.7) and the indirect ELISA was carried out. All of the incubation and washing steps were as described in Section 3.2.4. Briefly the antigen was titrated across an ELISA plate (NUNC; microtitre plate 96 U bottom wells) by doubling dilution. After the incubation and washing steps the plate was refilled with 50 μL/well of MAb E4 (4 mg/mL) diluted 1/8000 in TEN-TC buffer (Appendix 1.9), incubated at room temperature for one hour and then washed and tapped dry. The plate was refilled with affinity purified goat anti mouse

IgG conjugated to HRPO (Bio-Rad; Cat. No. 172-1011) diluted 1/2000 in TEN-TC buffer (Appendix 1.9). After another 1 hour incubation and a further washing step the reaction was developed by adding ELISA substrate (Appendix 1.10). The OD was read at the dual wavelengths of 415 and 490 nm using a plate reader (Pharmacia; Ultrospec III).

5.4 Identification of *Mycoplasma* Isolates

The identity of the *Mycoplasma* spp. isolates from Chapter 4 was determined by using an indirect ELISA (as described in Section 3.2.4) and purified MAb E4. Briefly, the *Mycoplasma* isolates were purified and sonicated as described in Section 4.2.5 for *M. bovis*. The protein concentration of each *Mycoplasma* spp. was adjusted to 0.5 mg/mL. A 96-well plate (NUNC; Cat. No. 167008) was coated in duplicate wells with each *Mycoplasma* sonicate diluted 1/100 in coating buffer (Appendix1.7). The plate was incubated overnight and then an indirect ELISA was carried out (Section 3.2.4).

5.5 Results

5.5.1 Immunisation

Before fusion, the mice were bled and the sera tested for antibody by indirect ELISA. The immunised mice that showed high antibody titres to *M. bovis* were used for fusion (Figure 5.1).

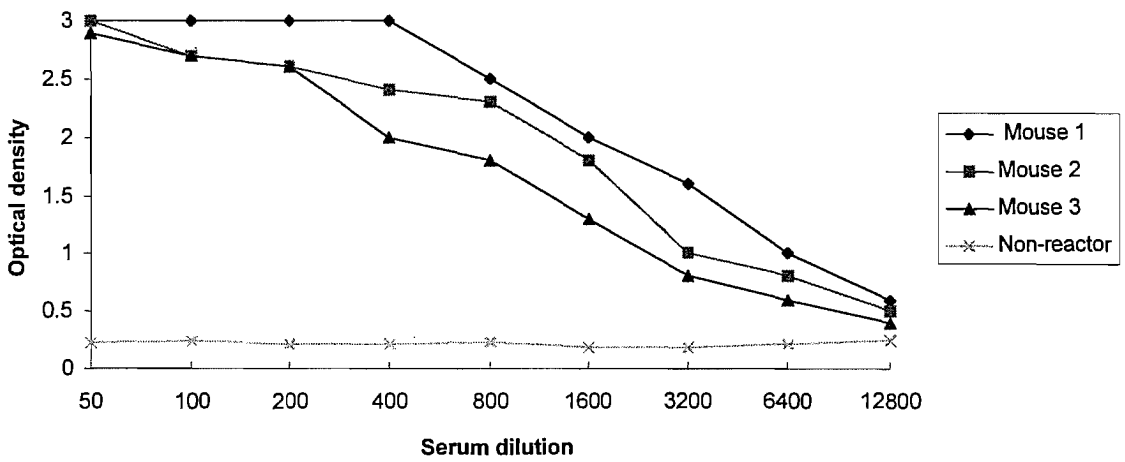

Figure 5.1 Antibody response detected by indirect enzyme linked immunosorbent assay in mice immunised with whole cell sonicated *Mycoplasma bovis* antigen.

5.5.2 Screening of hybridomas supernatants

There were 300 positive clones resulting from a single fusion with lymphocytes from mice immunised with whole cell sonicated *M. bovis* antigen. Fifteen clones of the original 300 hybridomas that appeared to actively secrete antibodies against *M. bovis* antigen were selected for further culture, and were subsequently transferred to 24 well plates and the supernatants screened.

The reaction of MAbs with whole cell sonicated antigen of *M. bovis* showed OD values of 2.0 at 1/800 dilution while the OD value with unsonicated antigen was 0.8. The hybridoma cells were frozen in liquid nitrogen and then thawed and cloned again. The supernatants of the hybridomas were tested again in an indirect ELISA. No differences in their reactivity were observed. This procedure was repeated three times and five stable, high producing hybridoma lines were selected for further study.

5.5.3 Affinity of monoclonal antibodies

The affinity of this panel of MAbs to *M. bovis* whole cell sonicated antigen is shown in Figure 5.2.

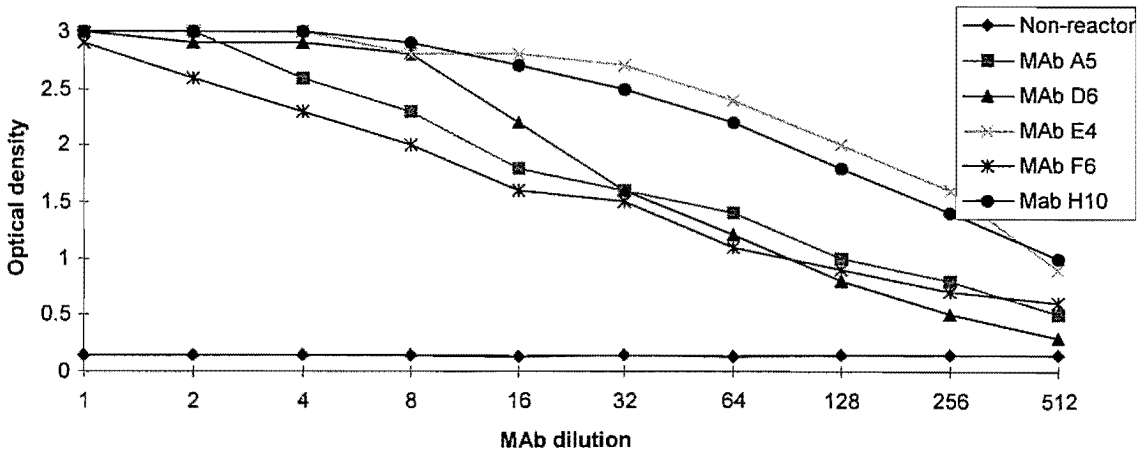

Figure 5.2 The affinity of five different monoclonal antibodies with *Mycoplasma bovis* whole cell sonicated antigen.

5.5.4 Specificity of monoclonal antibodies

The specificity of the MAbs from the five clones was determined using *Mycoplasma* species including: *M. arginini, M. agalactiae, M. bovirhinis, M.* group 7, *M. dispar, M. ovipneumoniae, M. bovigenitalium* and *M. bovis* in an indirect ELISA. While MAbs A5, D6, F6 and H10 showed some cross reactivity with *M. agalactiae, M. bovigenitalium, M. dispar* and *M. arginini*, the reactivity of MAb E4 was unique to *M. bovis* (Figure 5.3).

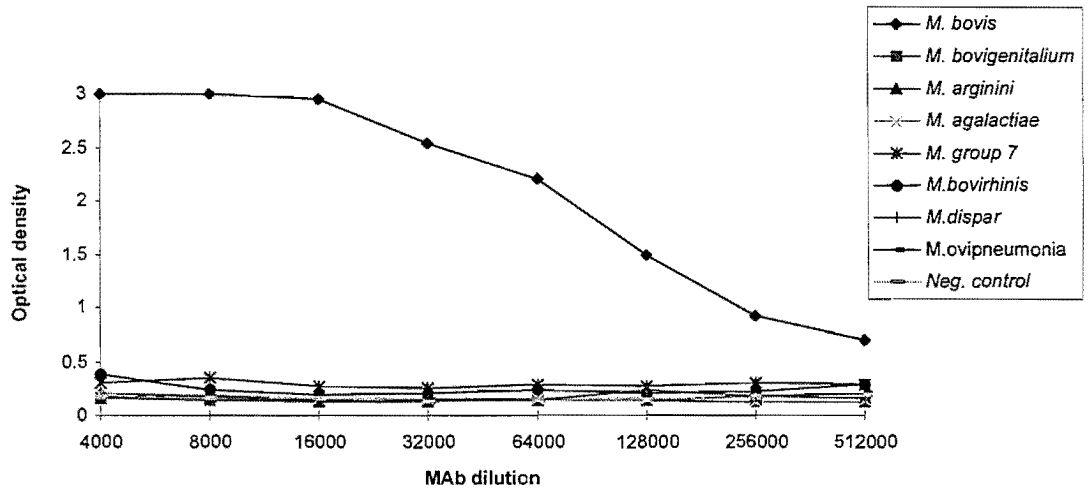

Figure 5.3 The specificity of monoclonal antibody E4 for whole cell sonicated antigen of different *Mycoplasma* species determined by indirect enzyme linked immunosorbent assay. The enzyme linked immunosorbent assay plate was coated with 0.2 µg of protein of each *Mycoplasma* antigen per well.

5.5.5 Western blotting

Based on the unique specificity of MAb E4 it was used for immunoblotting. The *Mycoplasma* polypeptides were separated on a 12% polyacrylamide gel. The SDS-PAGE profiles of whole cell proteins of *M. bovis* and *M. arginini* are shown in Figure 5.4. When transferred to a nitrocellulose membrane and blotted with MAb E4, two bands at 49 and 80 kDa were detected from two strains of *M. bovis*. No other *M. bovis* or *M. arginini* proteins reacted (Figure 5.5). There was no reaction of MAb E4 with *Mycoplasma* media.

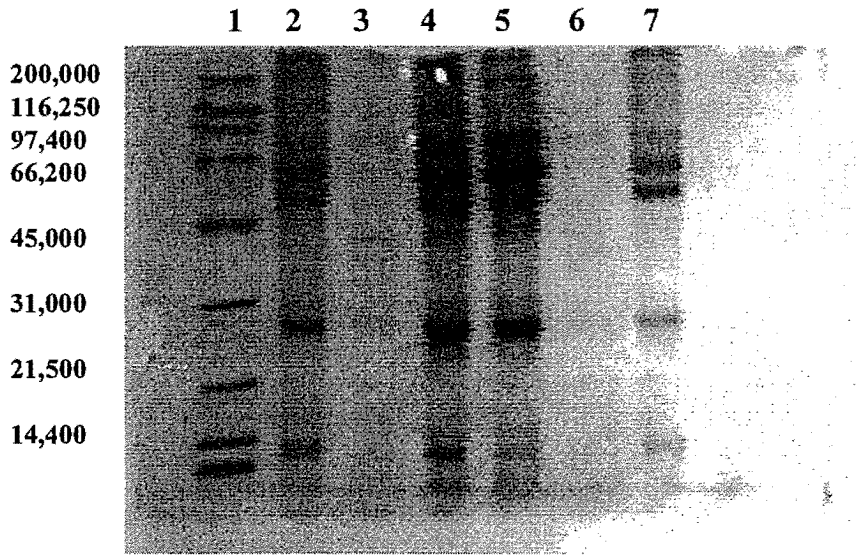

Figure 5.4 Sodium dodecyl sulphate polyacrylamide gel (12%) of whole cell lysate of *Mycoplasma bovis*, *Mycoplasma arginini* and three *Mycoplasma bovis* field strains stained with Coomassie blue. Molecular markers are indicated in kilodaltons.

Lane 1: Molecular weight standards
Lane 2: *M. bovis* 20 µg/well (PG-45 strain)
Lane 3: *M. arginini* 20 µg/well
Lane 4: *M. bovis* (local isolate) 20 µg/well
Lane 5: *M. bovis* (local isolate) 20 µg/well
Lane 6: *M. arginini* 10 µg/well
Lane 7: *M. bovis* (local isolate) 10 µg/well

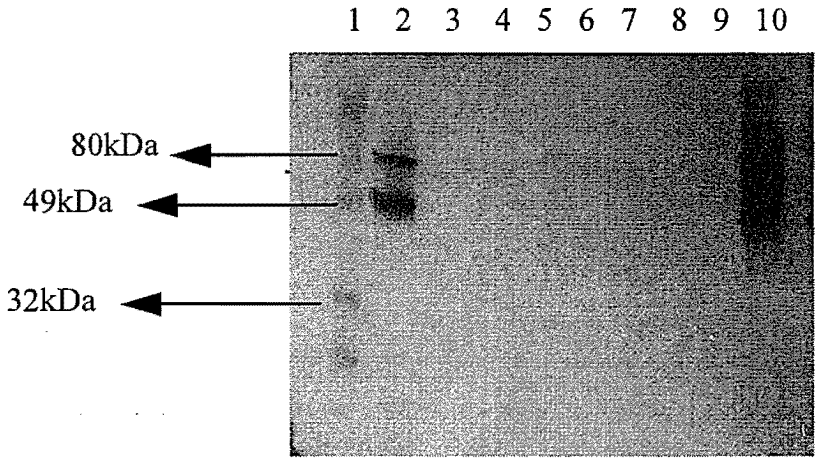

Figure 5.5 Immunoblot analysis of nine *Mycoplasma* species (reference strains) whole cell lysates (20 µg/well) were resolved on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. The gel was transferred onto nitrocellulose membrane and reacted with monoclonal antibody E4. Immunoblot was stained with phosphate buffered saline containing 3,3-diaminobenzidine tetrahydrochloride dydydrate and hydrogen dioxide until the desired colour intensity had developed.

Lane 1: Molecular weight standards
Lane 2: *M. bovis*
Lane 3: *M. arginini*
Lane 4: *M. agalactiae*
Lane 5: *M. bovigenitalium*
Lane 6: *M. bovirhinis*
Lane 7: *M. dispar*
Lane 8: *M. ovipneumoniae*
Lane 9: *M.* group 7
Lane 10: *M. bovis* (PG-45 strain)

5.5.6 Ascitic fluid production

The hybridoma E4 was grown in mice to produce ascitic fluid. Approximately 8 mL of ascitic fluid was collected from each mouse. The titre of the MAb E4 supernatant, that was OD 2.2 at dilution 1/100 (Figure 5.2), increased after ascitic fluid production to OD 3.0 (Figure 5.6), after purification to OD 2.8 (Figure 5.7) and after concentration to OD >3.0 (Figure 5.8).

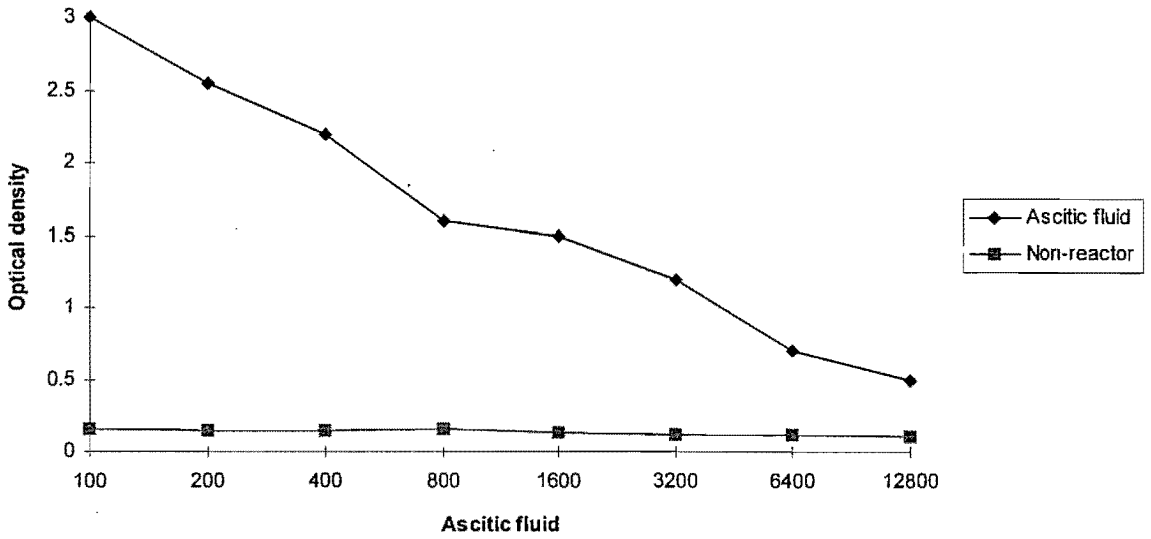

Figure 5.6 Hybridoma E4 ascitic fluid titration before purification. Whole cell sonicated *Mycoplasma bovis* antigen was used in an indirect enzyme linked immunosorbent assay configuration.

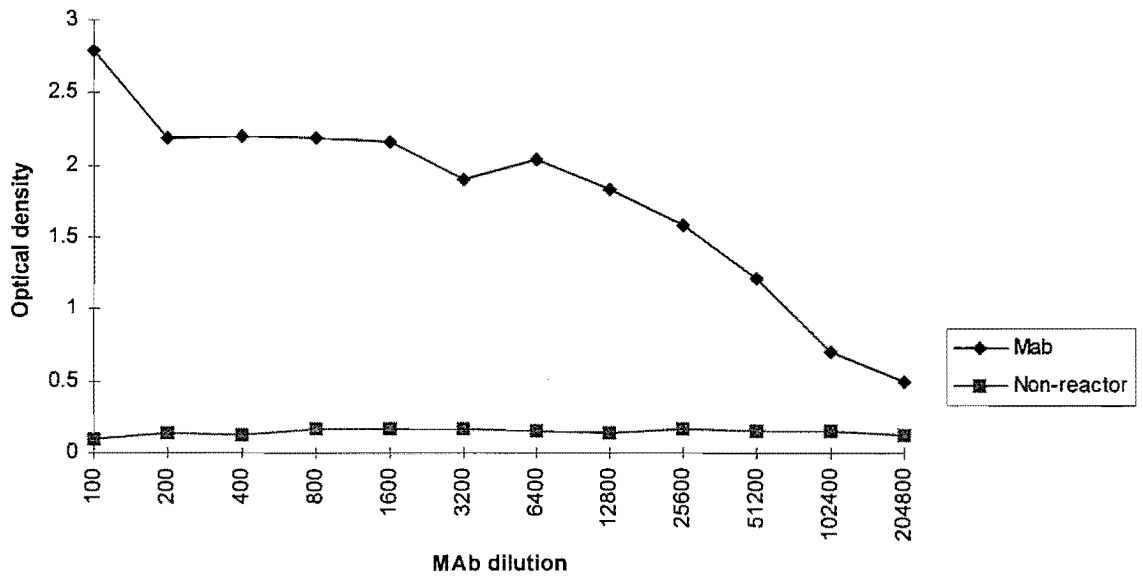

Figure 5.7 Hybridoma E4 ascitic fluid titration after purification. Whole cell sonicated *Mycoplasma bovis* antigen was used in an indirect enzyme linked immunosorbent assay configuration.

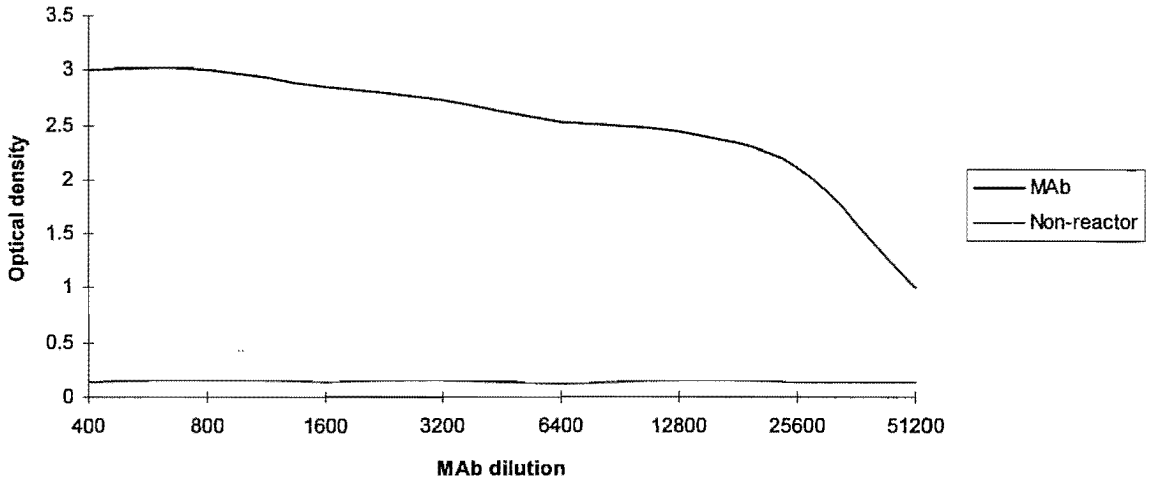

Figure 5.8 Hybridoma E4 ascitic fluid titration after purification and concentration. Whole cell sonicated *Mycoplasma bovis* antigen was used in an indirect enzyme linked immunosorbent assay configuration.

5.5.7 Sensitivity of MAb E4

The detection limit of MAb E4 (4 mg/mL) diluted 1/8000 was roughly 8 ng of *M. bovis* protein antigen in the indirect ELISA. As 2 µg of protein corresponds theoretically to $10^{7.3}$ bacteria (Thiaucourt et al. 1994) MAb E4 could theoretically detect 40 x 10^3 CFU of *M. bovis* per millilitre (Figure 5.9).

5.5.8 Isotypes of MAb E4

The immunoglobulin isotype of the MAb E4 from ascitic fluid was shown to be IgG_1 and Kappa (results not shown).

5.5.9 Conjugation

Monoclonal antibody E4 was conjugated to HRPO, but this resulted in a substantial drop in reactivity of the MAb E4 in the indirect ELISA from OD 3 to 0.8 after conjugation (data not shown).

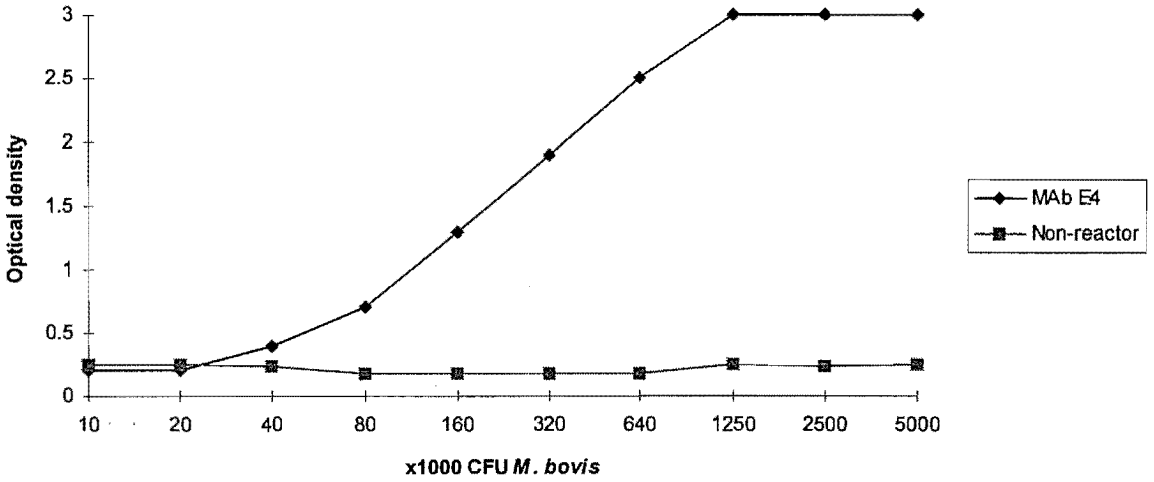

Figure 5.9 The sensitivity of monoclonal antibody E4 to limiting dilutions of known amounts of *Mycoplasma bovis* colony forming units per millilitre as determined by indirect enzyme linked immunosorbent assay.

5.5.10 Identification of *Mycoplasma* isolates

All *Mycoplasma* isolated from pneumonic lungs (Chapter 4) and one isolate from milk (Chapter 9) were identified as *M. bovis* (Figure 5.10) using MAb E4 in an indirect ELISA.

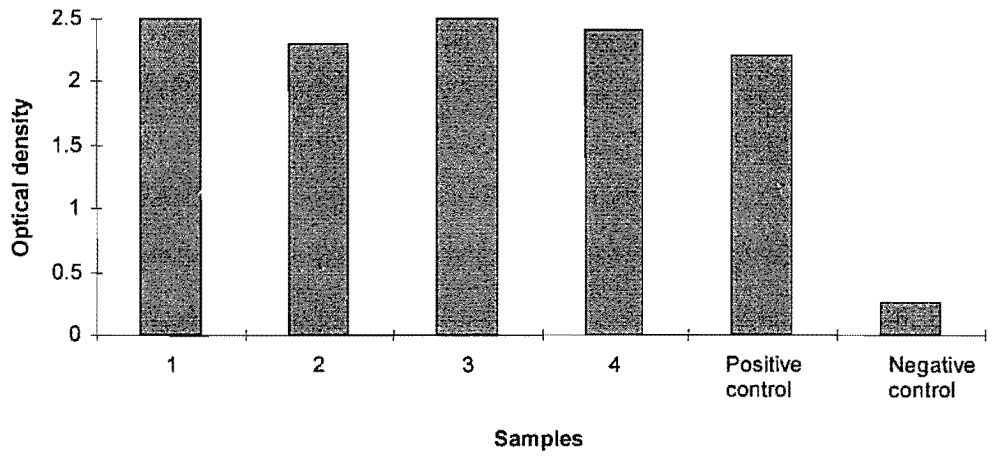

Figure 5.10 Identification of *Mycoplasma* isolated from cattle lungs (samples 1, 2, 3 described in Chapter 4) and mastitic milk (sample 4, as described in Chapter 9) using monoclonal antibody E4 in an indirect enzyme linked immunosorbent assay configuration. The positive control was *Mycoplasma bovis* reference strain and the negative control was *Mycoplasma arginini*.

5.6 Discussion

Monoclonal antibodies are unique in their specificity (Peter and Baron 1992). The main objective of this study was the production of a highly specific MAb to *M. bovis* with the capability of discrimination between *M. bovis* and other species of *Mycoplasma.* The use of such a MAb in an ELISA configuration may result in a higher degree of sensitivity and specificity. Monoclonal antibodies with reactivity to *M. bovis* have been reported (Berthold et al. 1992; Ball et al. 1994) but have shown cross reactivity with *M. agalactiae*.

Purified antigens should provide the basis for the immune system to respond to individual epitopes. The sonicated antigen used as an immunogen could potentially induce MAbs to all *Mycoplasma* epitopes. In this study, the MAbs produced showed high reactivity with sonicated whole cell *M. bovis* antigen in comparison with non-sonicated antigen. This probably suggests that these MAbs are against internal *Mycoplasma* proteins.

The antibody response of the immunised mice and the characteristics of the MAbs were detected initially by indirect ELISA. This finding suggests that the appropriate quantity and quality of antigen was used as immunogen. The fusion of myeloma cells with spleen cells produced 31% (300 out of 960) hybridomas. Of the 15 hybridomas frozen in liquid nitrogen, five stable lines (MAbs A5, D6, E4, F6 and H10) were cloned three times to remove any unstable cell types and reduce the possibility of cross reactivity with other bovine *Mycoplasma.*

The specificity of a MAb is essential for discrimination between species. The ability of purified MAb E4 to discriminate *M. bovis* from *M. arginini, M. bovirhinis, M. dispar, M. bovigenitalium, M.* group 7, *M. agalactiae* and *M. ovipneumoniae* was evaluated in an indirect ELISA. No cross-reactivity was seen in the ELISA, or by immunoblotting. In particular, it is important to note that MAb E4 does not cross react with *M. agalactiae*, as serological cross-reactivity between these two species has been reported previously (Poumarat et al. 1991; Berthold et al. 1992) and they are identical in cell shape, colony morphology and metabolic behaviour (Gummelt et al. 1996). This finding suggests that MAb E4 has potential for use in specific assays as it does not cross-react with other common bovine and ovine *Mycoplasma* species.

Mycoplasma bovis antigens recognised by MAb E4 were identified by immunoblotting. Monoclonal antibody E4 recognised the major proteins of *M. bovis* in the two strains investigated in this study. Despite heat and detergent treatment of *M. bovis* antigen before SDS-PAGE, the epitopes were still recognised by MAb E4.

To develop an ELISA that could detect *M. bovis* directly from biological samples, the conjugation of MAb E4 to HRPO was attempted. Unfortunately this resulted in a substantial drop in reactivity of MAb E4 in the indirect ELISA format (from OD 3 to 0.8). Thus no further investigation of conjugated MAb E4 was undertaken and secondary antibody conjugated to HRPO was used.

Determination of sensitivity of any assay is necessary to compare its value against existing assays. The sensitivity of MAb E4 in the indirect ELISA was 8 ng of *M. bovis* antigen protein estimated to correspond to 4 x 10^4 CFU of *Mycoplasma* (Thiaucourt et al. 1994). Although this sensitivity for detection of *Mycoplasma* might be regarded as low, it may be adequate for the detection of an active infection, even in poorly preserved samples. However the application of an antigen detection ELISA is restricted because it requires the sonication of *Mycoplasma* cells, which may lead to contamination of *Mycoplasma* antigen with proteins from the biological samples being tested.

CHAPTER 6

DEVELOPMENT OF A MONOCLONAL BLOCKING ENZYME LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODY TO *MYCOPLASMA BOVIS*

6.1 Introduction

Among the many serological tests developed, the indirect ELISA has been a sensitive, robust and broadly reactive assay compared with other methods for the detection of antibodies to *Mycoplasma* (Ansari et al. 1983; Talkington et al. 1985; Opitz and Cyr 1986). Unfortunately ELISAs for *Mycoplasma* have had problems with non-specificity resulting in cross reactivity among various *Mycoplasma* species (Onoviran and Taylor-Robinson 1979; Busolo et al. 1983; Thomas et al. 1987b; Ball et al. 1990). A MAb blocking ELISA configuration has shown better sensitivity and specificity than the indirect ELISA for the detection of antibodies in chickens to *M. gallisepticum* (Czifra et al. 1993, 1995), in pigs to *M. hyopneumoniae* (Feld et al. 1992) and in goats to *M.* F38 (Thiaucourt et al. 1994).

A blocking ELISA for antibody detection may be designed in such a way that the specificity of the assay is determined by the detector antibody rather than by the antigen. By use of a MAb in a blocking ELISA, achieving specificity at the epitope level is possible, even when crude antigen is used. In one example, serum from *M. hyopneumoniae* infected pigs could block the non-specific binding of a MAb (Feld et al. 1992). In this configuration, immunoglobulin from positive sera inhibits the MAb from attaching to its specific binding site and thus prevents colour development. In contrast, non-reactive sera will allow a strong colour reaction. It seems then that MAbs could provide a useful immunochemical tool for detecting antibodies to *M. bovis* in bovine sera in an appropriate ELISA configuration.

This chapter describes the development a monoclonal blocking ELISA with the aim of improving the detection of *M. bovis* specific antibody in cattle sera.

6.2 Materials and Methods

6.2.1 Serum sources

Serum samples ($n = 100$) were obtained from dairy herds on the Atherton Tableland, north Queensland. The polyclonal rabbit serum (PRS) to *M. bovis* (Section 3.2.3) was used as a positive reactive serum. Pre-immunisation rabbit serum (PRS) to *M. arginini* (Section 3.2.3) and foetal calf serum (FCS) were used as the negative reactive sera. The working dilution for the test cattle sera was standardised as undiluted, and diluted 1:2 and 1:10 in TEN-TC.

6.2.2 Blocking-ELISA

Blocking inhibition of MAb E4 by the PRS was studied with a modification of an indirect ELISA according to Saliki et al. (1993). All the incubation and washing steps were carried out as described in Chapter 3 (Section 3.2.4), with a few exceptions, and the working dilutions of the MAb and antigen were determined previously. Briefly, ELISA plates (NUNC) were coated with 0.5 μg/well of *M. bovis* whole cell sonicated antigen diluted in coating buffer (Appendix 1.7), sealed and incubated at room temperature overnight. The plate was washed three times with PBS-T and tapped dry. Aliquots of 50 μL/well of undiluted serum were added in duplicate wells to the antigen coated plate and the plate incubated for one hour at room temperature. The plate was washed three times with PBS-T and then tapped dry. The MAb E4 was diluted in TEN-TC and aliquots of 50 μL/well were added and allowed to react for one hour. The plate was washed with PBS-T and tapped dry. Binding of the MAb E4 was detected by the addition of goat anti-mouse HRPO conjugate and ELISA substrate as described in Section 5.2.7.

6.2.3 Quantification of MAb binding

Absorbance values of the undiluted test sera were compared with that of FCS used as the low-reactive serum, and the percent inhibition was calculated. Greater than 50% inhibition was considered to suggest a positive result, between 40% and 50% indicated an equivocal result, and less than 40% indicated a negative result. A test serum sample was considered negative in the blocking ELISA if it did not inhibit the binding of MAb E4 by more than 40%, compared with the controls and was probably the result of non-specific binding to the target epitope. The

development of the reaction was checked with controls in which no serum was added and others in which no MAb was added.

6.2.4 Calculation of percentage of inhibition

Samples were analysed in duplicate and ODs were read by a spectrophotometer at 415 nm and 419 nm (Bio-Rad model 3550 plate reader). In this assay the wells without MAb and serum were considered as 100% inhibition (OD_{100}) and the wells without primary serum (MAb alone) were considered as 0% inhibition (OD_0). Duplicate wells showing more than 10% difference in inhibition were re-tested, otherwise the result was the mean of the duplicate wells. The percentage inhibition (PI) for each sample was calculated by the following formula.

$$PI = \frac{(OD_0 - OD\ test)}{(OD_0 - OD_{100})} \times 100$$

6.3 Results

6.3.1 Standardisation of blocking ELISA

Serial dilutions of FCS were tested in competition with the MAb E4. As seen in Figure 6.1 the FCS, pre-immune rabbit serum and PRS to *M. arginini* did not inhibit binding of MAb E4. The greatest difference between negative and positive sera was observed with undiluted sera (Figure 6.1) and so undiluted sera were used in subsequent testing. The negative controls had percentages of inhibition lower than 10%, and the cut off value of 30% was chosen arbitrarily.

Of the 100 serum samples tested, 60 (60%) were positive by blocking ELISA (Figure 6.2).

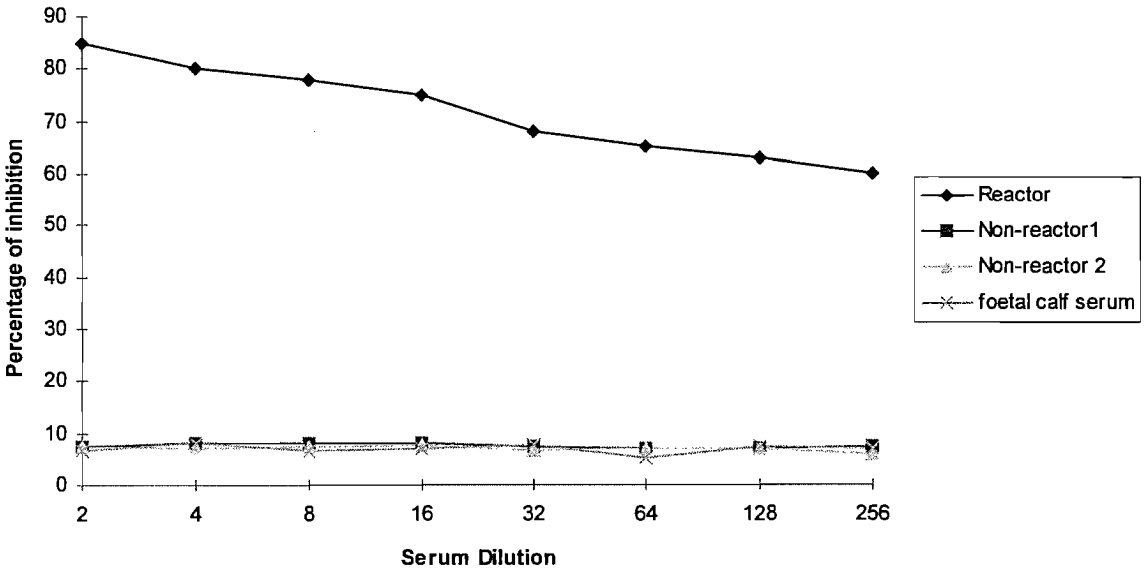

Figure 6.1 Graph demonstrating the percentage of inhibition of monoclonal antibody E4 binding by different dilutions of rabbit polyclonal antibody to *Mycoplasma bovis* (Reactor), foetal calf serum, pre-immune rabbit serum (Non-reactor 1) and polyclonal rabbit serum to *Mycoplasma arginini* (Non-reactor 2).

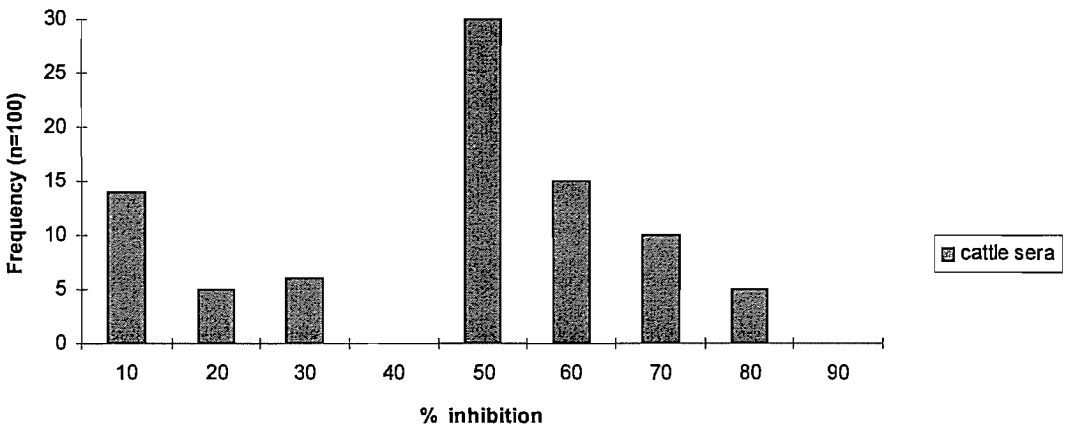

Figure 6.2 Distribution of reactor sera by percentage of inhibition in a blocking enzyme linked immunosorbent assay to detect *Mycoplasma bovis* antibody in 100 sera from dairy cattle from the Atherton Tableland, north Queensland.

6.4 Discussion

Antibody detection can provide information that may be used to assist with clinical diagnosis or it can be used to obtain meaningful information about the status of disease in a population (Burgess 1988). Growth of a pathogenic strain of *Mycoplasma* in an animal produces antibodies that can be detected by ELISA and immunoblotting, as well other serologic techniques (Ross and Young 1993). Unfortunately serodiagnostic methods for the diagnosis of some *Mycoplasma* infections currently show poor specificity because of their antigenic complexity (Kenny 1992). Development of an assay of improved specificity for the detection of *Mycoplasma* antibodies could have significant epidemiological and clinical application.

The purpose of the present study was to develop a sensitive and specific ELISA to overcome the problems associated with other serological tests. Traditional methods for reducing non-specific activity and cross-reactions in ELISA for *Mycoplasma* have included propagating *Mycoplasma* in serum-free medium (Avakian and Kleven 1990) or introducing a separate serum-absorption step before testing (Higgins and Whithear 1986). These attempts have not been completely successful and have increased the complexity of the test system.

A blocking ELISA offers an alternative method for the retrospective detection of *M. bovis* infection. By using MAb E4 with its unique specificity for *M. bovis,* a blocking ELISA was developed. The test was highly specific for *M. bovis* andbased on arbitrary cut off seemed to be sufficiently sensitive. Of the 100 serum samples tested in this study, 60% were positive. This suggests a high prevalence of *M. bovis* in the dairy cattle population tested. This result is consistent with the findings of others who used a blocking ELISA for the detection of antibodies to *M. gallisepticum* (Czifra et al. 1995), in which the detection rate in experimentally and naturally infected chickens were 84.7 and 72.6% respectively. This compared with 68.4 and 48.6% respectively for the haemagglutination inhibition (HI) test. It was concluded that the blocking ELISA is robust and the results reproducible, but further assessment is required to confirm its utility as a diagnostic ELISA.

CHAPTER 7

DEVELOPMENT OF A DNA PROBE FOR THE DETECTION *MYCOPLASMA BOVIS*

7.1 Introduction

The development of a sensitive and rapid laboratory diagnostic method for *Mycoplasma* infection is important to ensure the correct identification of the organism and for epidemiological studies. Current methods for the detection of *M. bovis* are restricted to culture and serology. Both methods are time consuming, laborious, difficult, expensive and false negative results are common (Simecka et al. 1992). Isolation and identification may take weeks, and few laboratories have the expert capability required to routinely culture *Mycoplasma.* Serodiagnosis requires the demonstration of increasing antibody titres ten to fourteen days after the onset of clinical symptoms (McCully and Brock 1992). Consequently, the infection cannot be detected during the incubation period and the early stages of the disease. Moreover, a critical problem is the serological cross reactions among *Mycoplasma* strains (Thirkell et al. 1991; Abdelmoumen and Roy 1995). The absence of practical and rapid detection methods and resistance to therapy therefore hamper the effective control of *Mycoplasma* infectious in cattle.

The development of a sensitive and specific assay for the detection of *M. bovis* is required (Simecka et al. 1992; Kirk and Lauerman 1994). A DNA hybridisation probe has been proposed as a supplement to current diagnostic methods owing to its high degree of specificity and the ability to detect early stages of infection (Hyman et al. 1989; Levisohn et al. 1989). Assays based on DNA hybridisation of *M. bovis* have been developed, but they have lacked complete specificity (Mattsson et al. 1991; McCully and Brock 1992; Hotzel et al. 1993).

In this chapter the preparation of a sensitive and specific DNA hybridisation probe for the detection of *M. bovis* is described.

7.2 Materials and Methods

The application of modern cloning technology to the study of *Mycoplasma* species requires a reliable method for the isolation of high molecular weight DNA from a small number of cells.

7.2.1 Bacterial isolates

The species of bacteria used in this study are listed in Table 7.1. Most of the *Mycoplasma* strains were obtained from National Collection of Type Cultures (Colindale, UK) and some strains were obtained from the Queensland Department of Primary Industry's Oonoonba Veterinary Laboratory, Townsville. Other bacterial strains were obtained from the then Department of Biomedical and Tropical Veterinary Science (now Department of Microbiology and Immunology, School of Biomedical and Molecular Science). Samples of bulk tank milk were obtained from Malanda Dairy Food, Malanda, Queensland. Individual cow milk samples were obtained from dairy herds on the Atherton Tableland, north Queensland.

7.2.2 *Mycoplasma* culture

A single colony of *M. bovis* was selected from MA, inoculated in 250 mL of MB (Section 4.2.1.3) in a 1 litre flask and incubated with constant agitation at 37°C, to a mid logarithmic phase of growth (as judged by the colour of the phenol red indicator in the medium) and harvested by centrifugation at 20000 *g* (Suprafuge 22 HFA, 22.50) for 30 minutes at 4°C. The pellet was washed twice with MWS (Appendix 1.1) to remove any the adhering MB protein.

7.2.2.1 Titration of viable Mycoplasma

The number of viable cells in the MB culture was determined by plate count. The MB culture was diluted and aliquots were plated on the surface of MA and then the number of colonies was counted to determine the number of CFU (Rodwell and Whitecomb 1983). Aliquots of other MB cultures were stored at -70°C until required.

Table 7.1 *Mycoplasma* and other bacterial species used in the development of the *M. bovis* specific probe.

Species	Strain
Mycoplasma bovis	NC10131[a]
Mycoplasma bovis	PG-45 (Donata strain)[b]
Mycoplasma ovipneumoniae	NC10151[a]
Mycoplasma agalactiae	NC10123[a]
Mycoplasma bovigenitalium	NC10122[a]
Mycoplasma bovirhinis	NC10118[a]
Mycoplasma dispar	NC101125[a]
Mycoplasma group 7	Leach N29 E Adelaide[b]
Mycoplasma bovigenitalium	PG-11 Ex Adelaide[b]
Mycoplasma arginini	WCA 7880[b]
Staphylococcus aureus	JCU[c]
Staphylococcus spp.	JCU[c]
Streptococcus uberis	JCU[c]
Streptococcus agalactiae	JCU[c]
Corynebacterium bovis	JCU[c]
Klebsiella pneumoniae	JCU[c]
Pseudomonas aeruginosa	JCU[c]
Pasteurella spp.	JCU[c]
Brucella abortus	JCU[c]
Yersinia enterocolitica	JCU[c]
Enterobacter cloacae	JCU[c]
Enterobacter faecalis	JCU[c]
Serratia marcescens	JCU[c]
Bacillus subtilis	JCU[c]
Escherichia coli	JCU[c]

a) Type strain NCTC, National Collection of Type Culture
b) Queensland Department of Primary Industry's Oonoonba Veterinary Laboratory, Townsville
c) Department of Biomedical and Tropical Veterinary Sciences (now Department of Microbiology and Immunology, School of Biomedical and Molecular Science)

7.2.3 Bacteria culture

A single colony of each bacterial strain listed in Table 7.1 was grown in 10 mL of brain heart infusion broth in a 200-mL flask, and harvested by centrifugation at 4000 *g* (Suprafuge 22 HFA, 22.50) for 15 minutes. The pellet was washed twice with PBS resuspended in PBS to approximately 10^9 cells/mL measured spectrophotometerically based on 1 OD_{600} =

10^9 cells/mL (Sambrook et al. 1989). Aliquots of the suspension were stored at -20°C until required.

7.2.3.1 Harvesting and lysis of bacterial cells

The washed pellet was resuspended in NTE buffer (pH 7.4) (Appendix 3.1) to approximately 10^9 cells/mL in a 1.5 mL microfuge tube, and lysed by adding SDS and proteinase K solution at a final concentration of 0.5% and 100 μg/mL respectively. The resultant solution was incubated in a water bath at 37°C overnight. The proteinase K solution was prepared by dissolving 100 mg of proteinase K in NTE buffer (pH 7.4) (Appendix 3.1) and incubated for 15 minutes in a 37°C water bath before adding to the cell suspension.

7.2.4 Preparation of Tris-buffered phenol

Saturated phenol was prepared according to the protocol described by Sambrook et al. (1989) with some modifications. Briefly, redistilled phenol (Progen, Australia) held at 4°C was warmed to room temperature and then melted at 55°C. To reduce oxidation of the melted phenol, hydroxyquinoline was added to a final concentration of 0.1% and then the mixture saturated with an equal volume of 0.5 M Tris-HCl pH 8.3 in a fume hood. The solution was mixed on a magnetic stirrer for 15 minutes and then allowed to settle until the aqueous and organic phases separated. The upper aqueous phase was removed using a glass pipette attached to a vacuum line equipped with traps. This procedure was repeated with the addition of equal volumes of 0.1 M Tris-HCl (pH 8.3) until the pH of the phenolic phase was 8.3. The final aqueous phase was removed, and to the equilibrated phenol, 0.2 volumes of 0.1 M Tris-HCl (pH 8.3) containing 0.2% β-mercaptoethanol was added. The solution was stored in a light-tight bottle at 4° C.

7.2.5 Purification of *Mycoplasma* and bacterial DNA

To remove any remaining residual cellular protein and also the proteinase K, the lysed cell solutions from Section 7.2.3.1 were treated with one volume of Tris buffered phenol. After adding phenol, the solution was gently shaken for 3 minutes and centrifuged at room temperature for 3 minutes at 10000 g (Microfuge E™). Without disturbing the interface, the upper phase was collected and poured into a clean tube. The solution was then mixed with

phenol-chloroform-isoamyl alcohol (10:9.6:0.4), shaken for 3 minutes and centrifuged for 3 minutes at 10000 *g* (Microfuge E™) at room temperature. The upper phase was removed and transferred to a clean tube. The solution was mixed with one volume of chloroform-isoamyl alcohol (24:1), shaken for 3 minutes and centrifuged for 3 minutes at 10000 *g* (Microfuge E™) at room temperature. The upper phase was removed to a clean tube and the DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 4.8) and two volumes of cold absolute ethanol. The solution was mixed by inverting several times and held at -70° C for 30 minutes. Most of the precipitated DNA was removed from the solution by winding onto a Pasteur pipette sealed at the tip and placed in a clean microfuge tube. The rest of the DNA was recovered by centrifugation at 10000 *g* (Microfuge E™) for 15 minutes at 4°C. The supernatant was discarded and the pellet of DNA was washed with 70% ethanol and repelleted by centrifugation at 10000 *g* (Microfuge E™) for five minutes and air dried. The pellet of DNA was dissolved in 200 µL Tris-EDTA (TE) buffer (pH 8.0) (Appendix 3.2) and treated with DNase-free RNase (Bresatec; Cat. No. AM-2286 RNase cocktail) at a concentration of 50 µg/mL in a 37°C water bath for 30 minutes. Following further phenol-chloroform extraction and ethanol precipitation the DNA was dissolved in 100 µL of TE buffer (pH 8.0).

7.2.6 Determination of DNA concentration

DNA concentration was measured spectrophotometrically (OD 260/280) as measured below (Sambrook et al. 1989) or, if at low concentration, from the intensity of fluorescence emitted by ethidium bromide.

7.2.6.1 Spectrophotometer method

The OD of the DNA solution was read at 260 nm using a spectrophotometer (Pharmacia; Ultrospec III). Ten microlitres of DNA solution were diluted in 40 µL of TE buffer (pH 8.0). The solution was dispensed into a 50-µL silica cuvette (Pharmacia, Sydney, Australia). The concentration of DNA in this solution was determined against TE buffer (pH 8.0) without added DNA as a blank and calculated on the basis that 50 µg of DNA/mL will have an OD_{260} of 1 (Sambrook et al. 1989).

7.2.6.2 Estimation by ethidium bromide fluorescence

In this method DNA was estimated by comparing the fluorescence of the sample with a series of DNA standards. As little as 1-5 ng of DNA can be estimated by this method. Briefly, the DNA sample was placed in one well of a U bottom ELISA plate (NUNC). A series of lambda DNA concentration standards (0, 1, 2.5, 5, 10 and 20 μg/mL) were prepared and 5 μL of each was placed in an ordered array in an ELISA plate. An equal volume of TE buffer (pH 8.0) containing 2 μg/mL ethidium bromide was added to each DNA sample in the ELISA plate and mixed well. The concentration of DNA was estimated by comparing the intensity of fluorescence in the sample with those of the standard solutions using a short wavelength ultraviolet illumination.

7.2.6.3 Agarose gel electrophoresis

The quality of the DNA preparation was evaluated as follows. One part of loading buffer (Appendix 3.3) was added to every five parts of DNA preparation (1 μg DNA) and then the samples were electrophoresed in 0.8% agarose gels (Progen) containing 0.01% ethidium bromide. The molecular weight markers were either *Hin*dIII digested lambda phage DNA (lambda/*Hin*dIII, Promega; Cat. No. G1711) or 1 Kb ladder(Gibco, BRL; Cat. No. 5675-010). A volume of 0.5 μL of a stock solution (0.67 μg/mL) was diluted in 9.5 μL TE buffer (pH 8.0) (Appendix 3.2). Electrophoresis was carried out at 80 V for about one hour. After electrophoresis the DNA was visualised and recorded using a Gel Doc 1000 system (Bio-Rad, California).

7.2.6.4 Determination of DNA fragment size

The migration of lambda /*Hin*dIII markers from the starting well was measured and the $\log_{10}$ of this distance was plotted against the known size of these DNA fragments. This standard curve enabled the interpolation of the size of the sample DNA band.

7.2.7 Restriction enzyme analysis of *Mycoplasma* DNA

The restriction enzyme analysis of *M. bovis* DNA was performed with restriction enzymes *Sau*3AI, *Eco*RI and *Hin*dIII. Briefly, the restriction digest solution was made up to a final

volume of 20 µL with sterile distilled water and allowed to incubate for two hours at 37°C. The following ingredients were added in order starting with the estimated volume of DNA solution, 2 µL of restriction enzyme buffer, 1 µL of BSA 1 mg/mL (Sigma Chemical Co.), and a volume of restriction enzyme to a final concentration of 8 IU/µg DNA. Digestion products were analysed by gel electrophoresis in 0.8% agarose gels containing 0.4 µg/mL ethidium bromide.

7.2.7.1 Extraction of DNA fragment from agarose gel

The whole digested genome of *M. bovis* was isolated from the gel and purified using a Gene clean kit (Bresatec) according to the manufacturer's instructions. Briefly, the gel was put in a sterile 1.5 mL microfuge tube and two volumes of sodium iodide (NaI) stock solution was added to one volume of the gel. The microfuge tube was incubated at 45°C in a water bath for five minutes. Five microlitres of GLASSMILK suspension were added to the solution and then centrifuged at 10000 *g* (Microfuge E™) for five seconds. The supernatant was discarded and the pellet was washed three times with 200 µL of ice cold NEW buffer. The supernatant was discarded and the pellet was resuspended in 50 µL TE buffer (pH 8.0). It was incubated at 45°C for 3-5 minutes and centrifuged at 10000 *g* for 30 seconds. The supernatant was placed in a clean tube and the concentration of *Mycoplasma* DNA fragments in the solution determined as described in Section 7.2.6.

7.2.8 Preparation of plasmid pUC19

7.2.8.1 Preparation of competent cells JM101

The *E. coli* strain JM101 was obtained from Bresatec. A single colony of *E. coli* JM101 from an overnight culture on 2YT agar was inoculated into a 3-litre flask containing 500 mL of 2YT broth (Appendix 3.4). The cells were grown at 37°C with vigorous shaking to an OD of 0.3 to 0.5 at a wavelength of 600 nm (Pharmacia; Ultrospec III). The cells were held on ice for 15 minutes, then centrifuged in a cold rotor at 4000 *g* for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 100 mL of ice-cold 0.1 M $CaCl_2$, then re-pelleted in a cold rotor at 4000 *g* for 10 minutes at 4°C. The supernatant was removed and the cells resuspended in 20 mL of ice-cold 0.1 M $CaCl_2$. The cell suspension was dispensed into microfuge tubes in 200 µL aliquots containing 20% glycerol and frozen in liquid nitrogen for

storage at -70°C. The quality of competent cells was tested by transformation with a known amount of plasmid DNA.

7.2.8.2 Transformation and propagation of plasmid DNA pUC19

Frozen competent cells prepared as described in Section 7.2.8.1 were used for transformation. One tube containing 200 µL of competent cells was taken from storage at -70°C. The cells were thawed by holding the tube in the palm of the hand. As the tube contents began to thaw the tube was put in an ice-bath for 10 minutes. The competent cells were transferred to a sterile, chilled 20 mL bottle. About 50 ng of pUC19 DNA (Promega) was added to the competent cells and the bottle was swirled gently several times and then placed on ice for 30 minutes. The bottle was transferred to a 42°C water bath for 90 second to heat shock the cells before removal to an ice bath. This was followed by adding 0.8 mL of 2YT. The mixture was incubated for one hour in a shaking incubator at 37°C to allow the bacteria to recover and express the antibiotic resistance gene. Volumes of 10, 50, 100, 200 µL of transformed competent cells (and untransformed competent cells as a control for transformation) were plated on 2YT agar plates containing 150 µg/mL ampicillin (Appendix 3.6). The transferred cells were gently distributed over the surface of the agar plates using a sterile bent Pasteur pipette. The plates were left open at room temperature under a laminar flow hood until dry and then incubated at 37°C. The rest of the transformed competent cells were stored at 4°C.

7.2.8.3 Plasmid DNA propagation

One ampicillin resistant colony from a fresh plate prepared as described in Section 7.2.8.2 was inoculated into 125 mL of 2YT broth containing 150 µg/mL ampicillin (2YTA) and shaken at 37°C overnight. The cells were pelleted by centrifugation at 4000 *g* (Suprafuge 22 HFA, 22.50) for 15 minutes at 4°C in a fixed angle rotor. The supernatant was discarded and the tubes were placed in an inverted position to allow all of the supernatant adhering to the tube to drain away. The bacterial pellet was resuspended in 25 mL of ice-cold STE buffer (pH 8.0) (Appendix 3.8). The bacterial cells were recentrifuged at 4000 *g* (Suprafuge 22 HFA, 2250) for 15 minutes at 4°C.

Plasmid DNA was isolated using an alkaline lysis procedure. The washed bacterial pellet derived from 125 mL culture was resuspended in 25 mL of glucose.Tris.EDTA (GTE)

solution (Appendix 3.9) to stabilise the bacterial cells. The solution was mixed and 250 µL of freshly prepared solution of lysozyme (Appendix 3.10) and 5 mL freshly prepared SDS lysis buffer (Appendix 3.11) were added. The contents were mixed by gently inverting the tube several times. The suspension was held at room temperature for 10 minutes and then 3.85 mL of ice cold potassium acetate solution (Appendix 3.12) was added to the suspension. The tubes were shaken gently several times, incubated on ice for 10 minutes and then centrifuged at 4000 *g* for 15 minutes at 4°C in a fixed angle rotor (Suprafuge 22 HFA, 22.50). The supernatant was filtered through four layers of cheese cloth into a 50-mL centrifuge tube. An equal volume of isopropanol was added, mixed well and incubated for 10 minutes at room temperature. The plasmid DNA was recovered by centrifugation at 5000 *g* for 15 minutes at room temperature in a fixed angle rotor (Suprafuge 22 HFA, 22.50). The supernatant was then carefully discarded and the open tubes were inverted to allow the last drops of supernatant to drain away. The pellet was washed with 70% ethanol at room temperature, the ethanol drained off and any beads of liquid that adhered to the walls of the tube were removed by a Pasteur pipette attached to a vacuum line. After all of the traces of ethanol in the pellets were removed, the pellet of DNA was dissolved in 750 µL of TE buffer (pH 8.0).

7.2.8.4 Purification of plasmid DNA

Plasmid DNA was purified by precipitation with PEG (Sambrook et al. 1989). Briefly, the plasmid DNA prepared as in Section 7.2.8.3 was transferred to a 2-mL microfuge tube and an equal volume of ice cold 5 M LiCl was added. The suspension was mixed well and centrifuged at 10000 *g* for 10 minutes at 4°C (Microfuge E™). The supernatant was then transferred into a new 2 mL microfuge tube. After adding an equal volume of isopropanol and mixing, the pellet of DNA was recovered by centrifugation at 10000 *g* for 10 minutes at room temperature (Microfuge E™). The supernatant was discarded and the resultant pellet was washed with 70% ethanol at room temperature. To dry the pellet, the open tube was placed on a pad of paper towels in the inverted position at room temperature until all of the visible traces of ethanol evaporated. The pellet was dissolved in 125 µL of TE buffer (pH 8.0) containing DNase-free RNase (Bresatec; Cat. No. AM-2286; RNase cocktail) at a final concentration of 20 µg/mL.

The plasmid DNA suspension was incubated at 37°C for 30 minutes and after the addition of 125 µL of 1.6 M NaCl containing 13% (w/v) PEG (PEG 6000), was mixed and the plasmid

DNA was recovered by centrifugation at 10000 *g* (Microfuge E™) for five minutes at 4°C in a microfuge tube. The supernatant was then removed by aspiration and the pellet was dissolved in 300 µL of TE buffer (pH 8.0).

The resultant solution was extracted once with phenol, once with phenol-chloroform and once with chloroform isoamyl-alcohol. Finally, the upper phase was transferred into a fresh tube, and the plasmid DNA was precipitated with the addition of 50 µL of 10 M ammonium acetate and 300 µL of cold ethanol. This mixture was incubated for 10 minutes at room temperature and then centrifuged at 10000 *g* (Microfuge E™) for five minutes at 4°C. The supernatant was discarded by aspiration, the DNA pellet rinsed with 300 µL of 70% ethanol and centrifuged at 4°C for two minutes. The supernatant was removed by aspiration and the open tube inverted on the bench until all of the visible traces of ethanol evaporated. Eventually the pellet was dissolved in 125 µL of TE buffer (pH 8.0) and the quality and concentration of plasmid DNA was determined as described in Section 7.2.6.

7.2.8.5 Restriction enzyme analysis of plasmid DNA

The closed, circular plasmid DNA (pUC19) was digested with *Bam*HI restriction enzyme (8 IU/µg plasmid DNA) in 10 x restriction buffer E (Promega; Cat. No. R602A) for two hours at 37°C in a water bath. A 0.5 µg amount of digested plasmid DNA was analysed by electrophoresis in a 0.8% agarose gel and the fragment sizes determined by comparison with *Hin*dIII digested lambda DNA size markers. The restriction enzyme was inactivated by heating the digested plasmid DNA to 68°C for 30 minutes.

7.2.8.6 Dephosphorylation of linearised plasmid DNA

To 1.0 µg of the linearised plasmid DNA (that was estimated to contain approximately 1.9 pmoles of 5'-terminal phosphate residues) 10 µL of 10x calf intestinal alkaline phosphatase (CIAP) dephosphorylation buffer (10 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_{22}$, 50 mM KCl, 50% (v/v glycerol) and 2.2 µL of CIAP (Promega; Cat. No. M1821) was added. The solution was mixed and incubated at 37°C for 15 minutes. The solution was then incubated for a further 45 minutes at 55°C following the addition of another aliquot (2.2 µL) of CIAP and then SDS and EDTA (pH 8.0) added to a final concentration of 0.5% and 5 mM, respectively. The solution was mixed well and proteinase K was added to a

final concentration of 100 μg/mL. The solution was incubated for 30 minutes at 56°C and then the CIAP was inactivated by incubation at 75°C for 10 minutes. The solution was made up to 300 μL by adding TE buffer (pH 8.0) and the DNA was extracted once with phenol and once with phenol:chloroform. The dephosphorylated plasmid DNA was precipitated with 10 μL of 10 M ammonium acetate and two volumes of ice-cold ethanol. The DNA was recovered by centrifugation at 10000 *g* (Microfuge E™) for 10 minutes at 4°C. The pellet was washed with 70% ethanol and centrifuged at 10000 *g* (Microfuge E™) for five minutes at 4°C. The supernatant was discarded by aspiration and, after evaporation of the ethanol, the dephosphorylated plasmid DNA was dissolved in 50 μL TE buffer (pH 8.0). The concentration of the dephosphorylated DNA was estimated as described in Section 7.2.6.

7.2.9 Cloning of the *Mycoplasma bovis* DNA into the plasmid DNA

About 50 ng of dephosphorylated plasmid DNA alone, 50 ng dephosphorylated plasmid DNA plus 50 ng completely digested of *M. bovis* DNA, 50 ng dephosphorylated plasmid DNA plus 100 ng completely digested *M. bovis* DNA and 50 ng of untreated plasmid (not dephosphorylated) were ligated following procedure described by Sambrook et al. (1989). Ligation reaction was performed in a total volume of 10 μL contain 10 × ligation buffer, 1 μL of 10 mM ATP, 1 μL of bacteriophage T4 ligase (1 weiss unit) (Promega Co., Cat # M181) and H_2O_2. Before ligation the dephosphorylated plasmid DNA was heated at 45°C for 5 minutes to allow fragment alinment with plasmid DNA. The ligation mixture was incubated over-night at 16°C. About 30% of each ligation mix was transformed with 200 μL of competent JM101 cells. Volumes of 10, 50, 100, 200 μL of transformed competent cells were plated on 2YT agar plates containing 150 μg/mL ampicillin, 40 μg X-Gal and 0.5 mM isopropylthiol-β-D-galactoside (IPTG)/mL (Appendix 3.7) and were gently distributed over the surface of the agar plates by using a sterile bent Pasteur pipette. The remaining transformed competent cells were stored at 4°C. The plates were held at room temperature under a laminar flow hood until dry and then incubated at 37°C. White and blue colonies appeared after 16 hours incubation. The remaining transformed competent cells were plated out on 2YT agar to give well-separated colonies. After incubation at 37°C the plates were double wrapped with cling film and stored at 4°C.

7.2.10 Immobilisation of the recombinant colonies

Hybond™-N+ positively charged nylon membrane (Amersham, UK) was used for lifting the blue and white colonies from the plates prepared as described in Section 7.2.9. Briefly, a circle of dry membrane was placed on the agar surface for one minute. To determine the orientation of the membrane, a sterile needle was used to mark around the edges of both the membrane and agar in an asymmetrical pattern. In addition the base of the Petri dish was marked with the same pattern using a marking pen. The membrane was removed from the plate by picking up its edge with a pair of blunt forceps and lifting from the same side used to lower the membrane onto the plate. It was placed colony contact side up on a Whatman filter paper to dry. To re-establish the colonies the plates were re-incubated for six hours at 37°C and then stored at 4°C until positive colonies had been identified from the hybridisation.

7.2.10.1 Fixation of the bacterial DNA to membranes

The DNA from the colonies was fixed to the membrane as follows. About 1.2 mL denaturing solution was poured onto a piece of plastic bench coat (Appendix 3.13) fixed to the bench. The dry membrane, with colony side up and numbered side down, was placed on the pool of denaturing solution and left for three minutes. The membrane was removed from the pad and placed on a clean Whatman paper to dry for three minutes. This procedure was repeated twice. The procedure was then repeated using a neutralising solution (Appendix 3.14) and the DNA was fixed. To fix the DNA the membrane was placed on 1.2 mL of 0.4 M NaOH for 20 minutes, and dried for three minutes. Finally the membrane was washed in 5 x SCC for less than one minute, dried on clean Whatman paper, wrapped in cling wrap and stored at 4°C.

7.2.11 Colony hybridisation

7.2.11.1 Prehybridisation

The membranes were placed on a nylon-mesh membrane that was pre-wetted in 2 x SSC (Appendix 3.15) and then treated with 10 mL prehybridisation solution containing 1% SDS and 10% dextran sulfate in a hybridisation tube. It was incubated with constant agitation for six hours at 65°C in hybridisation oven.

7.2.11.2 Preparation of radioactive probe

The random priming system (GIGA prime labelling kit, Bresatec; Cat. No. GPK-1 K 081) was used for radiolabelling the *M. bovis* DNA, according to the manufacturer's recommendations. Briefly, about 200 ng of whole genomic DNA of *M. bovis* was placed in a 1.5 mL sterile microfuge tube. The DNA was denatured by heating at 95 °C for five minutes, quenched on ice, centrifuged for five seconds to bring down condensation, and then the following mixture was added (to a total volume of 24 µL). Six microlitres of decanucleotide solution, 6 µL of nucleotide/buffer cocktail, 5 µL (50 µCi) of ^{32}P-dATP and 1 µL enzyme solution. The solution was centrifuged at 10000 *g* for a few seconds and mixed by gently tapping the tube. The reaction was incubated at 37 °C for 10 minutes and then inactivated by heating at 65 °C for 10 minutes.

The ^{32}P-labelled DNA was separated from unincorporated ^{32}P-dATP by using a Sephadex G-50 column. A 0.8 x 12-cm column was prepared by suspending 0.5 g of Sephadex G-50 medium in 20 mL column buffer [10 mM Tris-HCl (pH 8.0), 0.01 mM EDTA, 0.1% SDS and 5 mM β-mercaptoethanol]. The column was equilibrated with column buffer that was allowed to run off and the beads allowed to settle without becoming completely dry. The reaction mix was applied directly to the column and eluted with 5 mL of column buffer. Aliquots (0.3 mL) of eluate were collected in microfuge tubes, and monitored for radioactivity as counts per second (cps) using a Geiger counter. Based on cps of each fraction, two peaks were observed. The contents of three tubes from the first peak with high cps were combined, mixed with 100 µg denatured salmon sperm DNA and then added to the tube of pre-hybridisation solution containing the membranes. The probe was prepared before the completion of pre-hybridisation.

7.2.11.3 Hybridisation

The probe was used at a concentration 10 ng/mL of prehybridisation buffer. To this, one mL hybridisation solution containing 100 µg denatured salmon sperm DNA and denatured radioactive probe prepared as described in Section 7.2.11.2 was added to the tube containing pre-hybridisation solution prepared as described in Section 7.2.11.1. The lid was tightened and sealed with parafilm to prevent any possible leakage, and the incubation was continued with constant agitation for a further 18 hours at 65 °C.

7.2.11.4 Post hybridisation (washing step)

The membranes were washed twice with 100 mL of 2 x SSC at room temperature for five minutes with constant agitation, twice with 200 mL of a solution containing 2 x SSC and 1% SDS at 65°C for 30 minutes with constant agitation, and then twice with 100 mL of 0.1 x SSC at room temperature for 30 minutes with constant agitation.

7.2.11.5 Screening the colony hybridisation

An intensifying screen (Amersham, Hyper cassette) and X-ray film (Fuji film) were used to screen the colony hybridisation. The membrane was wrapped in cling wrap and then placed in a cassette. In the dark room X-ray film was placed on top of the membrane. The film was generally exposed for 18 hrs at -70°C. The cassette was removed from -70°C, and in the dark room the film was removed immediately from the cassette and developed by soaking the film at room temperature in X-ray developer (Ilford) diluted one in four with tap water for three minutes, then X-ray fixer (Ilford) diluted one in four with tap water for three minutes, and then washed in running water for five minutes. The film was dried at room temperature.

7.2.12 Alignment of probe-positive colonies with colonies on original plates

The positive colonies were identified on the film and aligned with the membrane and original plates held at 4°C. Fourteen white colonies showing high intensity activity were selected. Each recombinant colony was removed using a tooth pick, inoculated in 125 mL 2YTA broth (Appendix 3.6) in a 500-mL flask, and incubated at 37°C overnight with vigorous shaking. The DNA from the 14 colonies was extracted as described in Section 7.2.8.4 and stored at -20°C.

7.2.12.1 Size fractionation of recombinant DNA

The size of the cloned fragments was determined by restriction digestion of recombinant DNA with *Eco*RI and with *Hin*dIII as described in Section 7.2.8.5. The fragments were electrophoresed in a 1.6% agarose gel containing 0.4 µg/mL ethidium bromide. The size of the fragments was determined as described in Section 7.2.6.4.

7.2.12.2 Purification Mycoplasma bovis DNA fragments

Mycoplasma bovis DNA fragments were retrieved from recombinant plasmid DNA by two restriction enzyme digests. Ten micrograms of each recombinant plasmid DNA was digested with *Eco*RI and *Hin*dIII in a total volume of 180 µL of solution containing 18 µL of Multicore restriction buffer, 8 µL of BSA solution (2 mg/mL), 8 IU/µg DNA of each restriction enzyme and 141 µL distilled water. The preparation was mixed and incubated in a 37°C water bath overnight. A 0.5 µg amount of digested DNA was electrophoresed to check the digestion was complete. If this electrophoresis indicated a successful digestion the whole of the digested recombinant DNA was electrophoresed in 1.6% agarose gel (using a 4-cm comb) at 100 V for 45 minutes. Each DNA insert visualised using long wavelength UV light was excised from the gel with a disposable razor blade. The gel slice was put onto sterile Whatman filter paper placed in a microfuge tube that has a hole pierced in the base. This microfuge tube was placed in a larger microfuge tube that was placed in a 10-mL tube. The DNA fragment was removed from the gel by centrifugation at 3000 *g* for five minutes. The resultant solution containing the DNA fragment was precipitated with 0.1 volume of 3 M sodium acetate and one volume isopropanol as described in Section 7.2.8.6. The DNA fragment was dissolved in 50 µL TE buffer (pH 8.0), and the concentration was determined spectrophotometrically before storage at -20°C.

7.2.13 Dot blot hybridisation

Five hundred nanograms of chromosomal DNA from different species of bovine *Mycoplasma* or an equal amount of DNA from other bacteria (Table 7.1) were used for dot blot hybridisation. The DNA was denatured by heating at 90°C for five minutes, then held on ice for 30 seconds before addition of an equal volume of 20 x SSC. Nylon membrane (Hybond-N +Amersham) was marked in a grid pattern with a pencil, soaked in 10 x SSC and then air dried. The entire DNA preparation was spotted on the nylon membrane strips using approximately 5 µL aliquots and the membrane allowed to dry between each aliquot. The membranes were moistened with the DNA side up with 2 mL of denaturing solution (Appendix 3.12) on absorbent pads, for five minutes and then placed onto absorbent pads moistened with 2 mL neutralising solution for one minute. The membranes were air dried and the DNA was fixed to the membrane by incubating at 80°C for 2 hours.

A single membrane was used for each probe. Pre-hybridisation was carried out in a 10-mL solution containing 1% SDS and 10% dextran sulfate with constant agitation for six hours at 65°C. Hybridisation was conducted overnight in the same solution with the addition of 1 mL hybridisation solution containing 100 mg denatured salmon sperm DNA per millilitre and 200 ng random-primed ^{32}P-labelled probe. The membranes were then washed twice (five minutes for each wash) in 100 mL of 2 x SSC, four times in 200 mL of 2 x SSC plus 1% SDS at 65° C, and twice in 100 mL of 0.1% SSC at room temperature (30 minutes for each wash) with constant agitation.

The radiolabelled probes were detected by autoradiography with Fuji X-ray film for 18 hours at -70°C as described in Section 7.2.10.4. The results were quantified on a Bio-Rad Gel-Doc system and recorded as OD units.

7.2.14 Milk samples

Once the conditions for hybridisation were established for the detection of *M. bovis* grown from artificial media, it was used for detection of *M. bovis* in commercial milk samples. Bulk milk samples listed in Table 7.2, from 10 commercial dairy herds in north Queensland, were obtained from Malanda Dairy Foods, Malanda, north Queensland. Commercial milk, mixed with 10^9 CFU of *M. bovis*, was used as a positive control and commercial milk without adding *M. bovis* was used as a negative control. Composite milk samples from individual cows (127 samples) provided from one dairy farm on the Atherton Tableland were also tested in the hybridisation protocol.

Table 7.2 Farm details provided with bulk milk samples obtained from Malanda Dairy Foods.

Farm No	Type of cow	Approx. location
1	Friesian	Millaa Millaa
2	Ayrshire/Friesian Jersey/Holstein	Yungaburra
3	Jersey/Friesian	Millaa Millaa
4	Mixed Holstein Illawarra	Millaa Millaa
5	Friesian/Jersey	Kairi
6	Friesian	Evelyn Central
7	Holstein	Butcher Creek
8	Jersey/Holstein	Butcher Creek
9	Holstein	Butcher Creek
10	Unknown	Unknown

7.2.15 Extraction of DNA from milk

A 300-µL volume of milk was treated overnight with an equal volume of NTE buffer (pH 7.4) containing SDS and proteinase K at a final concentration 0.5% and 100 mg /mL respectively. The DNA was extracted as described in Section 7.2.5.

7.3 Results

About 200 mg of cells was produced from 250 mL *Mycoplasma* culture. These cells yielded 70 µg genomic DNA. The *M. bovis* DNA was shown to be pure when visualised by agarose gel electrophoresis. Restriction enzyme digests of *Mycoplasma* genomic DNA with *Sau*3AI gave complete digestion of DNA (Figure 7.1).

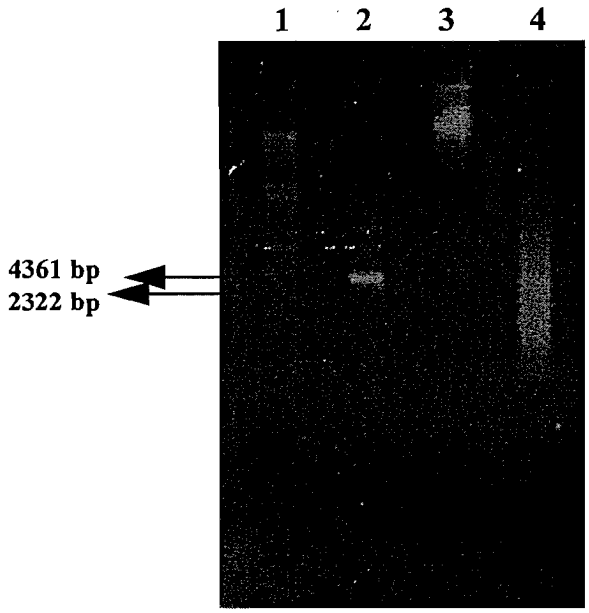

Figure 7.1 Agarose gel electrophoresis of genomic *Mycoplasma bovis* whole DNA.
Lane 1: MWM (lambda DNA/*Hin*dIII)
Lane 2: Plasmid pUC 19
Lane 3: Undigested whole genomic *M. bovis*
Lane 4: Restriction enzyme digestion of whole genomic *M. bovis* DNA with *Sau*3AI.

The bands produced by restriction digestion of *M. bovis* DNA ranged from approximately 0.1 to 3 Kb and were able to be purified from the gel. Because the compatibility of DNA digested with *Sau*3A and the *Bam*HI site of pUC19, the *Mycoplasma* DNA fragments were able to be cloned into the *Bam*HI site (Figure 7.2). Overall the efficiency of transformation of competent cells using the $CaCl_2$ procedure was ~3 x 10^5 colonies/µg plasmid DNA.

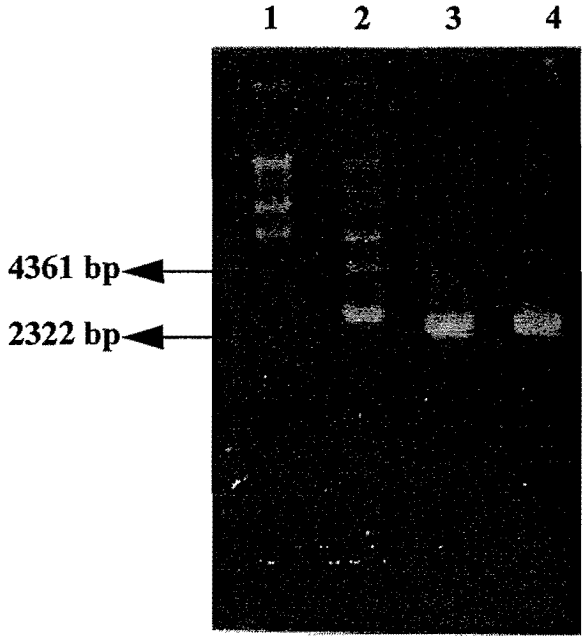

Figure 7.2 Agarose gel electrophoresis of restriction enzyme digestion of plasmic pUC19 with *Bam*HI.
Lane 1: MWM (lambda DNA/*Hin*dIII
Lane 2: Undigested pUC19
Lane 3 & 4: Plasmid pUC19 digested with *Bam*HI.

7.3.1 Cloning test

The tube containing dephosphorylated pUC19 DNA without *M. bovis* DNA produced no blue colonies from 10 μL, and three blue colonies from 100 μL of reaction mixture confirming the success of dephosphorylation of pUC19 as described in Section 7.2.8.6. The tube containing dephosphorylated plasmid DNA and 50 ng of *M. bovis* DNA produced eight white colonies and five blue colonies per microlitre of transformed reaction mixture on 2YTA agar. Cloning of *M. bovis* fragments into plasmid DNA was improved by increasing the relative *M. bovis* DNA concentration. The tubes containing dephosphorylated plasmid and 100 ng of *M. bovis* fragments DNA produced 12 white colonies and four blue colonies per microlitre of reaction mixture on 2YTA agar.

In the tube containing linearised pUC19 without CIAP treatment, the plasmid could circularise and express antibiotic resistance. Thus over 100 colonies were produced from 10 μL, and 800 colonies from 100 μL, of transformed reaction mixture. Transformation of competent cells with 20 ng of circular plasmid DNA (the positive control) produced too many blue colonies to count, indicating the efficiency of transformation.

7.3.2 Identification of a specific probe

Following colony hybridisation 14 white colonies which hybridised strongly to the *M. bovis* genomic DNA probe were selected randomly for detailed study (Figure 7.3).

Restriction enzyme digestion of selected recombinant pUC19 plasmids with either *Eco*RI or *Hin*dIII are shown in Figure 7.4 and Figure 7.5 respectively. Double digestion of recombinant plasmids with *Eco*RI and *Hin*dIII resulted in linearised plasmids and a number of *M. bovis* DNA fragments (Figure 7.6) of different molecular size.

Figure 7.3 Colony hybridisation of recombinant colonies using *Mycoplasma bovis* genomic DNA as a probe. A strong signal of hybridisation of white colonies were obtained with the probe in comparison with the blue colony as a negative control.

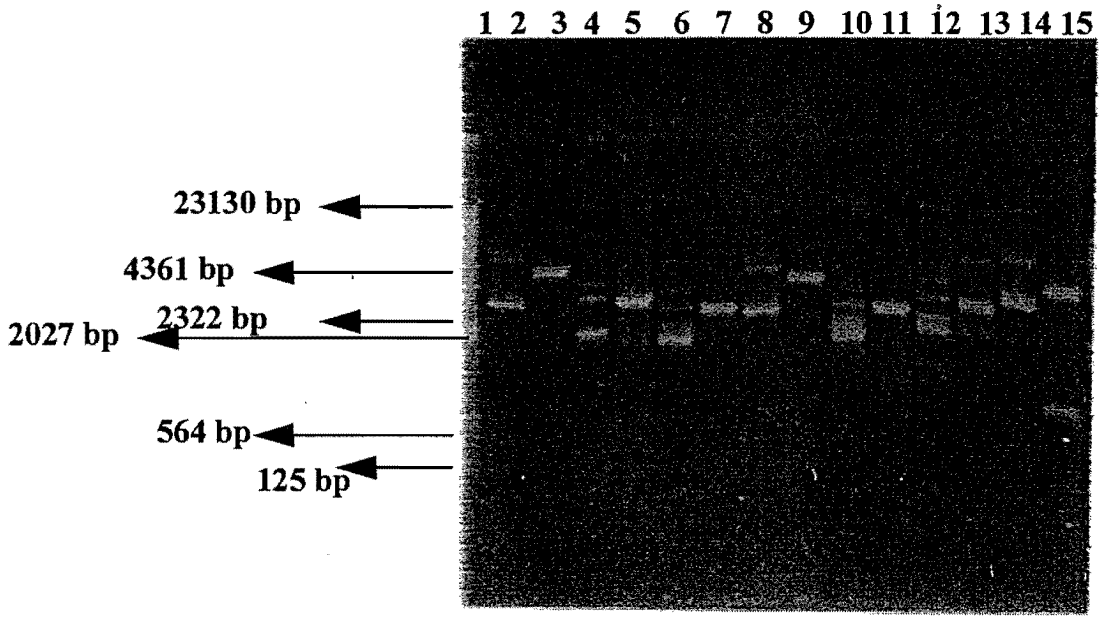

Figure 7.4 Agarose gel electrophoresis of selected recombinant pUC19 plasmids containing *Mycoplasma bovis* DNA using *Eco*RI.

Lane 1: MWM (lambda DNA/*Hin*dIII)
Lane 2: Uncut R (colony 2-2)
Lane 3: Cut R (colony 2-2)
Lane 4: Uncut R (3-4)
Lane 5: Cut R (3-4)
Lane 6: Uncut R (1-4)
Lane 7: Cut R (1-4)
Lane 8: Uncut R (3-1)
Lane 9: Cut R (3-1)
Lane 10: Uncut R (5-4)
Lane 11: Cut R (5-4)
Lane 12: Uncut R (2-1)
Lane 13: Cut R (2-1)
Lane 14: Uncut R (1-1)
Lane 15: Cut R (1-1)

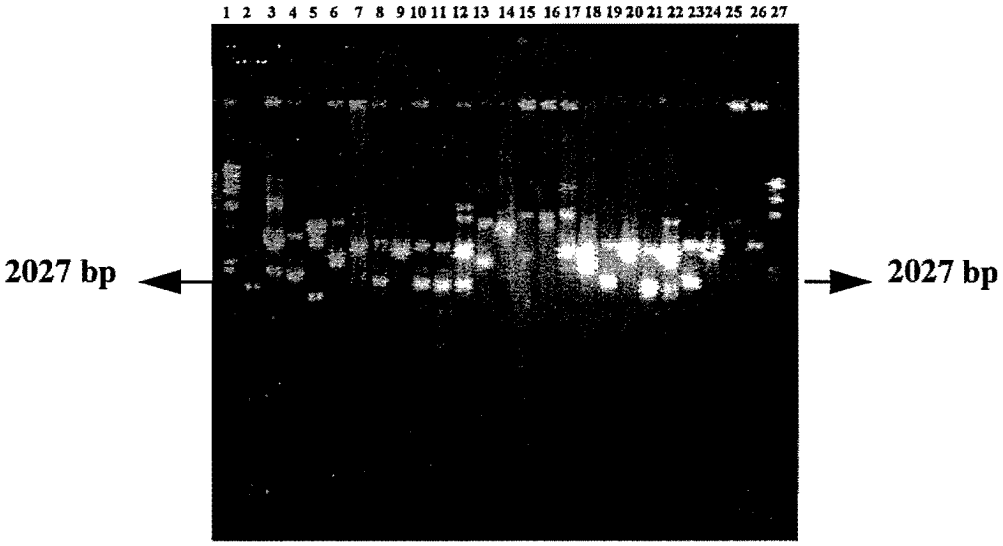

Figure 7.5 Agarose gel electrophoresis of selected recombinant (R) plasmids digested with *Hin*dIII.

Lane 1: MWM (lambda DNA/*Hin*dIII
Lane 2: Uncut R (colony 3-2)
Lane 3: Cut R (colony 3-2)
Lane 4: Uncut R (4-4)
Lane 5: Cut R (4-4)
Lane 6: Uncut R (2-2)
Lane 7: Cut R (2-2)
Lane 8: Cut R (3-4)
Lane 9: Uncut R (3-4)
Lane 10: Uncut R (1-1)
Lane 11: Cut R (1-1)
Lane 12: Uncut R (8-4)
Lane 13: Cut R (8-4)
Lane 14: Cut R (2-4)
Lane 16: Uncut R (1-7)
Lane 17: Cut R (1-7)
Lane 18: Uncut R (1-4)
Lane 19: Cut R (1-4)
Lane 20: Uncut R (1-6)
Lane 21: Cut R (1-6)
Lane 22: Uncut R (3-1)
Lane 23: Cut R (3-1)
Lane 24: Uncut R (5-2)
Lane 25: Cut R (5-2)
Lane 26: Plasmid pUC19
Lanes 27 & 28: MWM (lambda DNA/*Hin*dIII)

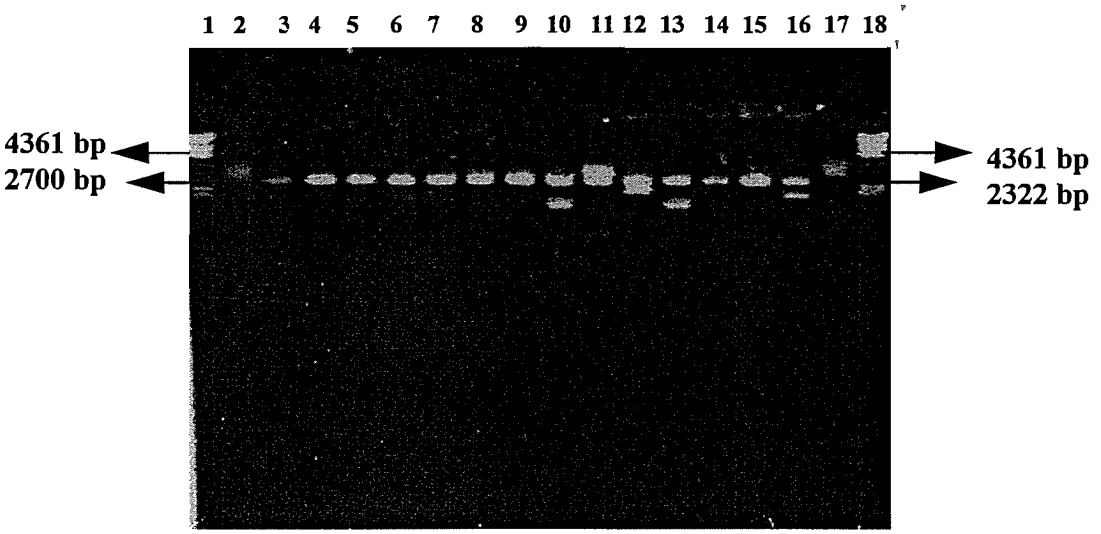

Figure 7.6 Agarose gel electrophoresis of recombinant (R) plasmids digested with both *Eco*RI and *Hin*dIII.

Lane 1: MWM (lambda DNA *Hin*dIII)
Lane 2: MWM (100 bp ladder)
Lane 3: R (colony 1-1)
Lane 4: R (colony 2-1)
Lane 5: R (1-6)
Lane 6: R (2-4)
Lane 7: R (2-4)
Lane 8: R (2-2)
Lane 9: R (5-2)
Lane 10: R (3-1)
Lane 11: R (4-4)
Lane 12: R (5-4)
Lane 13: R (3-4)
Lane 14: R (8-4)
Lane 15: R (1-4)
Lane 16: R (1-3)
Lane 16 & 17: MWM (100 bp ladder)
Lane 18: MWM (lambda DNA/*Hin*dIII)

The number of fragments from each recombinant plasmid depended on the number of restriction sites present in the DNA insert. Using double digestion 16 fragments ranging in size from 0.1 to 2 kb were detected. In all cases, the 1 kb ladder, or lambda *Hin*dIII, uncut DNA and non-recombinant plasmid DNA were used as markers. The size and number of fragments from selected clones are tabulated in Table 7.3.

Table 7.3 Size and numbers of fragments produced by restriction enzyme digestion from selected clones.

Recombinant	Fragments from *Eco*RI Cut	Fragments from double cut *Eco*RI & *Hin*dIII
	(Size of fragments in kb)	
R (1-7)	4.5, 1.8	1.8, 1.2, 0.7, 2.7
R (1-1)	5.0	0.8, 1.6, 2.7
R (1-2)	4.1, 0.8	1.5, 0.8, 2.0
R (6-4)	4.8	2.20, 2.7
R (2-2)	4.7	2.0, 2.7
R (3-1)	4.4, 0.1	0.1, 1.7, 2.7
R (8-4)	4	0.5, 0.7, 2.7
R (5-2)	3.4	0.8, 2.7
R (2-4)	3.3	0.7, 2.7
R (3-2)	3.3	0.7, 2.7
R (5-4)	3.1	0.4, 2.7
R (4-4)	3	0.3, 2.7
R (1-6)	3	0.3, 2.7
R (1-4)	2.9	0.2, 27

7.3.3 Dot blot hybridisation

Of 16 fragments purified from the gel, 12 fragments ranging in size from 0.1 to 2.0 kb were selected and used to prepare probes for dot blot hybridisation. Probes *Mb* 8-4 (0.7 kb), *Mb* 1-1 (1.6 kb), *Mb* 3-1 (1.7 kb), *Mb* 1-7 (1.2 kb) and *Mb* 2-2 (2.0 kb) reacted strongly with the *M. bovis* reference strain and *M. ovipneumoniae* and reacted moderately with *M. arginini*, *M. agalactiae, M. dispar* and *M. bovigenitalium* (Figure 7.7). These probes had no cross reactivity with *M. group 7* and *M. bovirhinis*. Probe *Mb* 1-4 (0.2 kb) and *Mb* 3-1(0.1 kb) failed to hybridise with *M. bovis* DNA, because of unstability in hybridisation temperature. Probes *Mb* 5-4, *Mb* 8-4, *Mb* 4-4, *Mb* 5-2 were found to hybridise with *M. bovis* and *M. ovipneumoniae*. The probe *Mb* 1-6 reacted with two strains of *M. bovis* only (Figure 7.7 and Figure 7.8). It did not hybridise with DNA from other microorganisms including:

Staphylococcus aureus, Staphylococcus spp., *Streptococcus uberis, Streptococcus agalactiae, Corynebacterium bovis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Brucella abortus, Yersinia enterocolitica, Enterobacter cloacae, Enterobacter faecalis, Pasteurella* spp., *Serratia marcescens, Bacillus subtilis* or *Escherichia coli.*

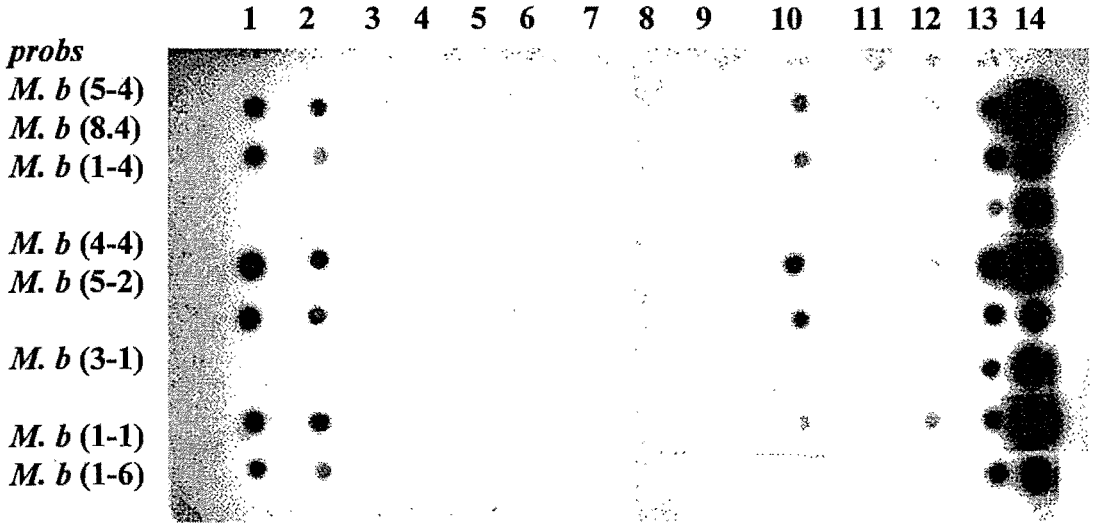

Figure 7.7 Autoradiograph of a dot blot hybridisation of different *Mycoplasma bovis* probes with whole genomic DNA from different *Mycoplasma* species.

Lane 1:	*M. bovis* Ref*
Lane 2:	*M. bovis* QDPI[+]
Lane 3:	*M. agalactiae* Ref
Lane 4:	*M. dispar* Ref
Lane 5:	*M. bovirhinis* Ref
Lane 6:	*M. bovigenitalium* Ref
Lane 7:	*M. group* 7 QDPI
Lane 8:	*M. bovigenitalium* QDPI
Lane 9:	*M. ovipneumoniae* Ref
Lane 10:	*Mycoplasma* spp. (local isolate)
Lane 11:	*M. arginini* QDPI
Lane 12:	pUC19 (negative control)
Lane 13:	Recombinant pUC19 (positive control)

* Indicates use of the reference strain

[+] Queensland Department of Primary Industry's Oonoonba Veterinary Laboratory, Townsville

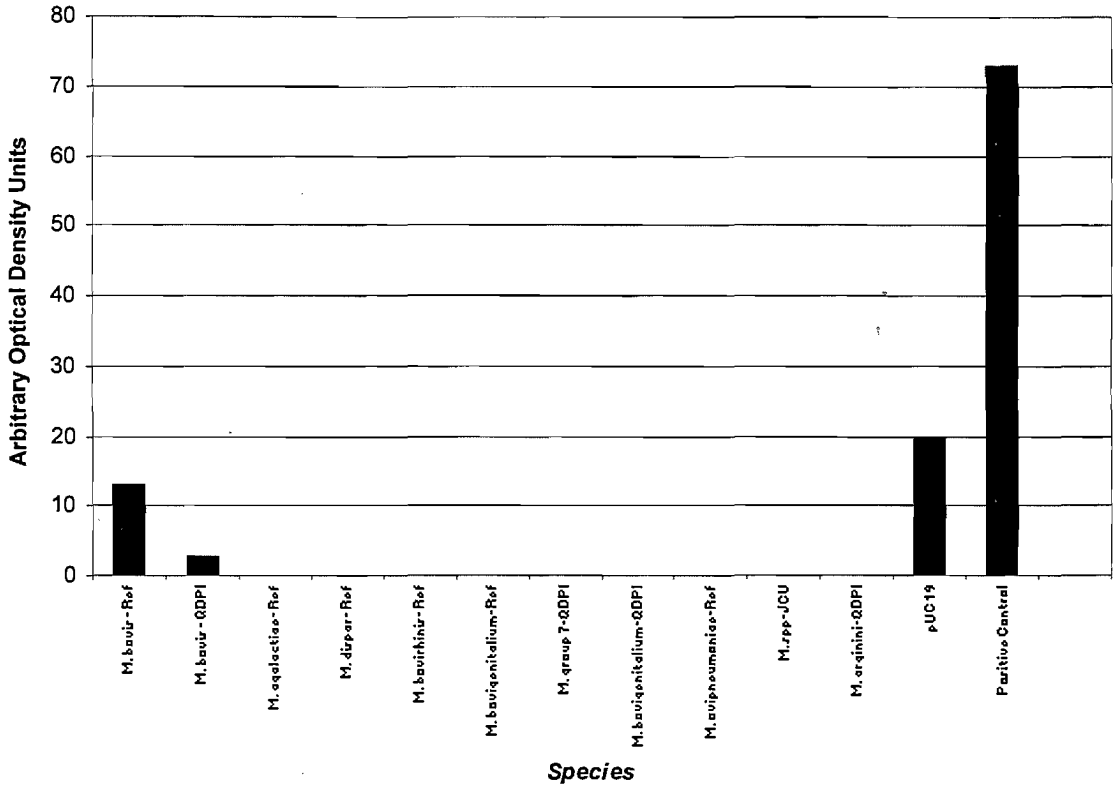

Figure 7.8 Bar graph prepared by scanning the X-ray film obtained during the dot blot procedure using the Bio-Rad Gel Doc system and associated software. Five hundred nanograms of chromosomal DNA from different species of mycoplasma were immobilised on to nitrocellulose membrane and hybridised with proble *Mb* 1-6. The arbitrary optical density units are an expression of the total area of each dot combined with the density. Care was taken not to overexpose the X-ray film and to avoid reciprocity failure. The molar representation of the pUC19 vector and the recombinant plasmid from which the probe was derived is far in excess of genomic DNA. "Ref" indicates use of the reference strain, QDPI refers to strains obtained from the Queensland Department of Primary Industry's Oonoonba Veterinary Laboratory, Townsville and JCU refers to an isolate obtained from the James Cook University's bacterial culture collection.

Hybridisation studies of bulk milk from dairy herds on the Atherton Tablelands indicated that nine of 10 herds were infected with *M. bovis.* The hybridisation assay results are summarised in Figure 7.9. At a herd level, the hybridisation assay revealed that 59% (75 out of 127) of cows were infected with *M. bovis* (Figure 7.10).

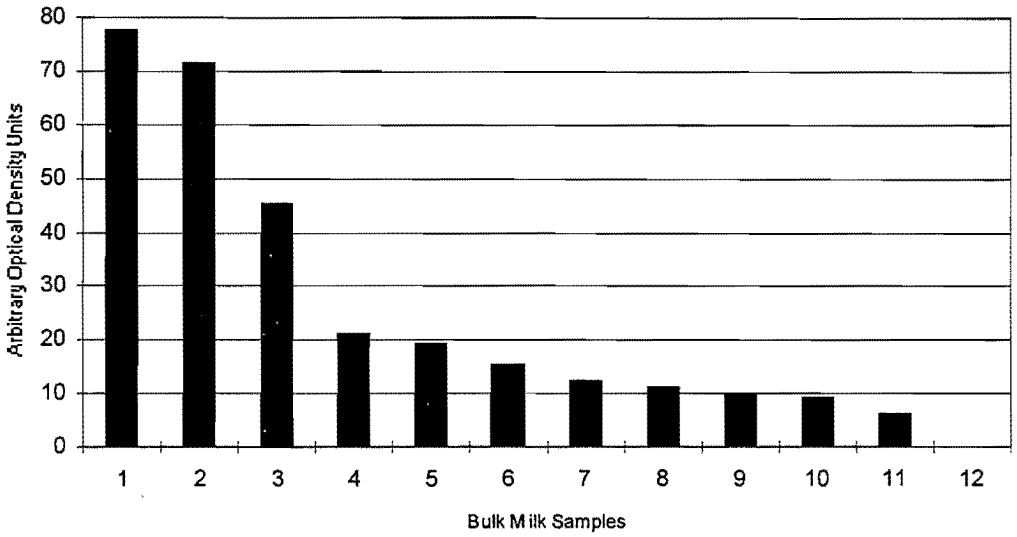

Figure 7.9 Bar graph prepared by scanning the X-ray film obtained during the dot blot procedure for the presence of *Mycoplasma bovis* using bulk milk samples. Commercial milk mixed with 10^9 colony forming units of *Mycoplasma bovis* was used as a positive control (sample 4) and commercial milk without added *Mycoplasma bovis* was used as a negative control (sample 8).

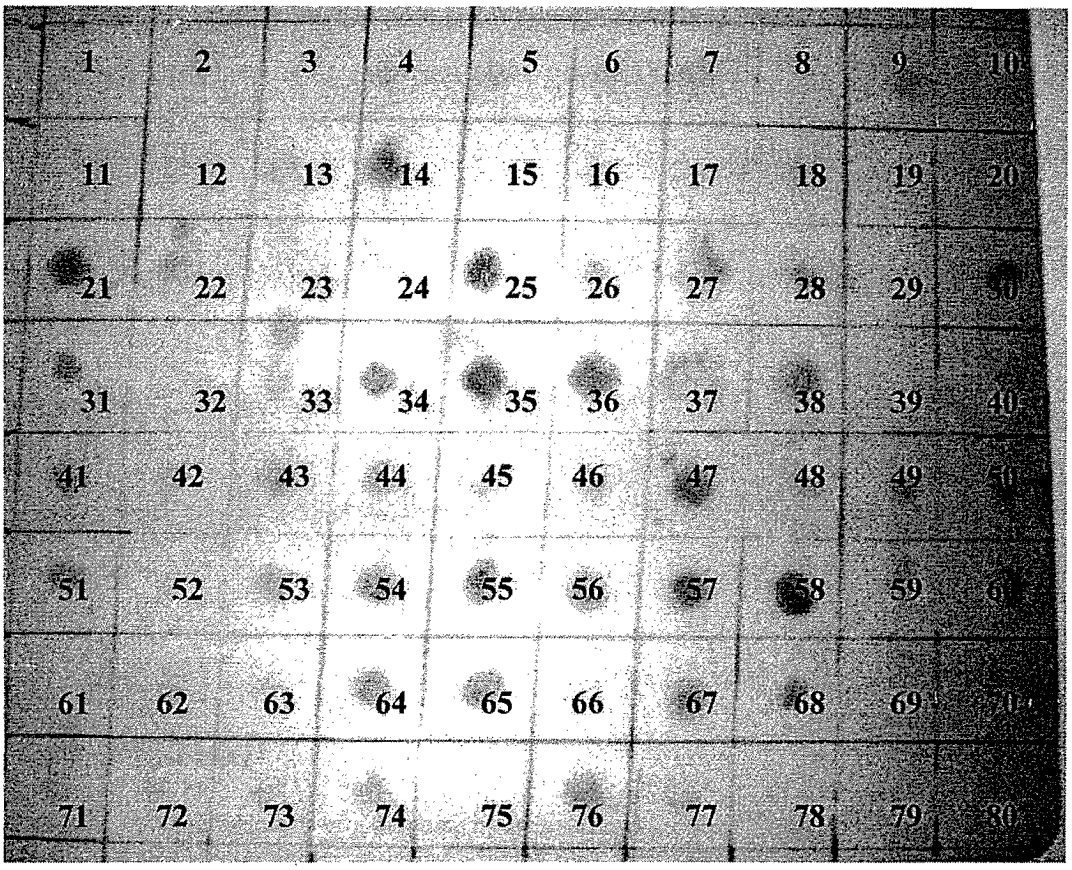

Figure 7.10 Autoradiograph of a dot blot hybridisation of DNA of 80 milk samples from individual cows using the probe *Mb* 1-6.

7.3.4 Sensitivity of the probe

To check the sensitivity of the probe *Mb* 1-6, the uninfected milk sample (number 12), which was found to be negative by dot blot hybridisation, was seeded with known amounts of *M. bovis*. Dot blot hybridisation was carried out (Figure 7.11 and Figure 7.12) to determine the minimum detection limit in CFUs/mL of milk.

The detection limit of this assay was roughly 200 CFU/mL. As can be seen in Figure 7.11 the OD from the radiograph of the dot blot from experimentally infected milk probed with *Mb* 1-6 probe increased with increasing numbers of *M. bovis* in the milk.

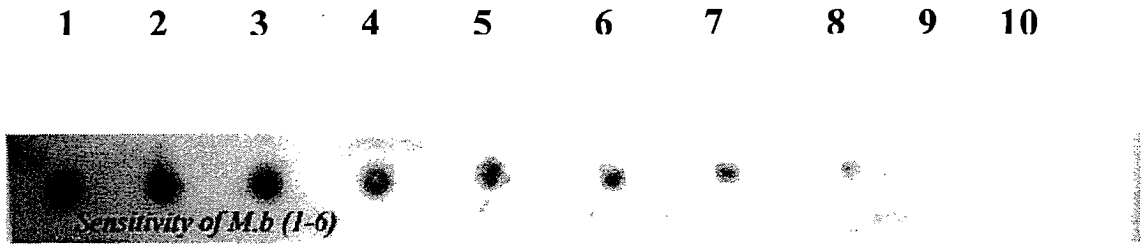

Figure 7.11 Autoradiograph of a dot blot hybridisation of alpha ^{32}P dATP labelled *Mb* 1-6 probe against DNA from experimentally infected bulk milk sample number 12 with various colony forming units of *Mycoplasma bovis*.
Number 1-10: 4500 x 10^3, 450 x 10^3, 45 x 10^3, 4.5 x 10^3, 450, 290, 200, 100, 0 colony forming unit *M. bovis*/mL respectively.

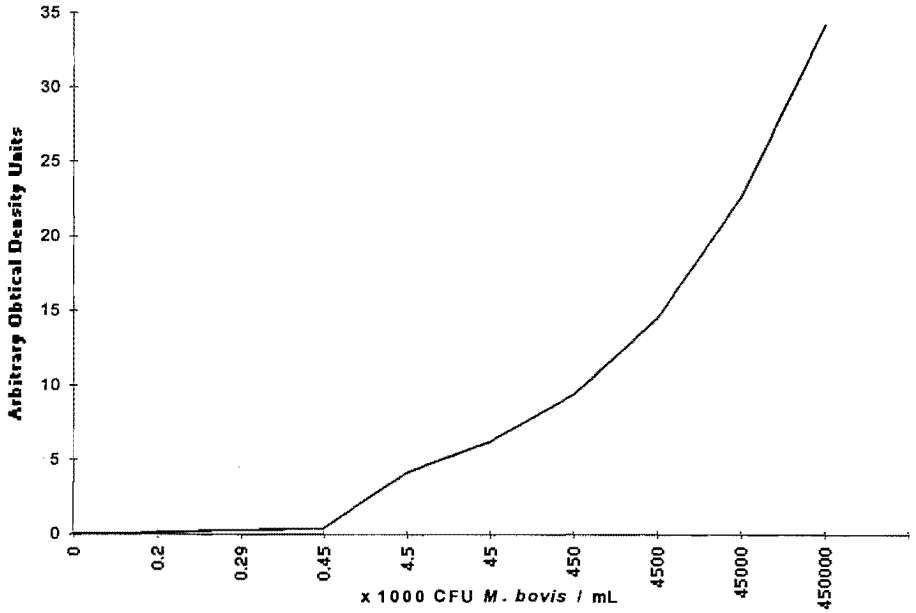

Figure 7.12 Known numbers of colony forming units of *Mycoplasma bovis* were added to a known negative bulk milk sample (Number 12 from Figure 7.9) and the milk samples were subjected to dot blot hybridisation with *Mb* 1-6 probe. The resultant arbitrary optical density units are recorded for each sample.

7.4 Discussion

Current methods used to diagnose *M. bovis* infections are inadequate because they lack speed, sensitivity and specificity (Simecka et al. 1992). Since DNA probes can overcome these problems (Razin et al. 1987; Kirk and Lauerman 1994), it was decided to design a specific DNA probe to discriminate between *M. bovis*, other *Mycoplasma* species and other bacterial species commonly found in cattle. Total DNA from *M. bovis* was prepared from a late-exponential phase culture, and used to make a genomic library. The *Mycoplasma* DNA was completely digested and the fragments generated were purified from the gel and ligated to the plasmid vector that had been linearised and dephosphorylated. Frozen competent cells were used for transformation with the ligated plasmid mixture and recombinant clones were cultured on suitable agar plates containing appropriate antibiotic indicators.

Colony hybridisation with whole *M. bovis* genomic DNA was carried out to find a recombinant colony showing strong intensity signals indicating the present of a suitable DNA

fragment. The hybridisation was performed in the presence of dextrane sulfate SDS and salmon sperm to block the non-specific reaction of the probe with the surface of the filters. The dextran sulfate increases probe concentration and as a result, the sensitivity and hybridisation rates (Mason et al. 1990).

Following colony hybridisation 14 recombinant colonies with high intensity signals were selected from the plate, re-grown, and their DNA extracted and double digested with *Eco*RI and *Hin*dIII. The fragments generated were purified from the gel and used in dot blot hybridisation. The method used for purification of the *M. bovis* fragments in this study has an advantage over other methods (Sambrook et al. 1989) in terms of simplicity and cost. As reported by other workers (Mayfield et al. 1990) large fragments may show more non-specific reactions than small fragments, and therefore large fragments were avoided.

The washing step in any hybridisation assay is of paramount importance (Razin et al. 1987). To reduce any background hybridisation the stringency of washing was increased. Stringent conditions were necessary to obtain high specificity of the probe due to a high level of DNA homology between *M. bovis* and the other *Mycoplasma* species, especially *M. ovipneumoniae* as found in this study. Although it has been reported that DNA homology among *Mycoplasma* species is low (Sugina et al. 1980), in this study high homology with other *Mycoplasma* species was found.

Following dot blot hybridisation of the library with *M. bovis* chromosomal DNA a DNA clone (1-6) was selected. The specificity of probes constructed from this clone was checked against DNA extracted from *M. bovis* and other *Mycoplasma* species. The probe *Mb* 1-6 was further investigated for its potential as a hybridisation probe after it was found to be stable, sensitive and specific for *M. bovis*. DNA probes made from this clone were investigated for their potential as hybridisation probes. It was found that the probe was both sensitive and specific for the detection of *M. bovis*.

Hybridisation was carried out with the two *M. bovis* strains as well as vector DNA and the recombinant plasmid from which the *M. bovis* DNA fragments were derived. The negative control (vector DNA) was bound because the probe DNA contained short stretches of the multiple cloning site. In addition, the molar representation of these hybridisation regions in

the vector and recombinant plasmid DNA is much higher than that of the *M. b* (1-6) complementary sequence in the same ng quantity of *Mycoplasma* DNA.

Determination of the sensitivity of any assay is necessary to compare its value against existing assays, and to assess whether it meets sensitivity requirements. The sensitivity of a DNA probe depends on the concentration of the DNA and size of the dot blots on the membrane (Razin et al. 1987). The sensitivity of the probe *M. b* (1-6) was excellent when tested in a dot blot assay against serial dilutions of *M. bovis*. Low levels of *Mycoplasma* (200 CFU/mL) that may be present during the incubation period or in chronically-diseased animals would be able to be detected.

The specificity of a DNA probe is particularly important for diagnostic usage. The probe developed in this study only gave a strong reaction with *M. bovis* (Reference strain) and reacted moderately with *M. bovis* (Oonoonba strain). It did not cross react with other *Mycoplasma* species. Moreover the probe did not cross react with other microorganisms likely to be found in association with bovine clinical and subclinical mastitis.

The specificity of the probe is of crucial importance for use under field conditions. In particular, it is important to note that the probe does not cross react with *M. agalactiae*, as genomic (Mattsson et al. 1991) and serological cross-reaction between these two species has been reported (Erno and Stipkovits 1973; Boothby et al. 1981; Poumarat et al. 1991; Berthold et al. 1992).

The interference of proteins, fats and ions present in the milk may cause false positive and false negative results (Hotzel et al. 1993). In this study no background problems were encountered with biological samples, however there was a direct correlation between the number of *M. bovis* (CFU) in the milk and the OD of the dots obtained with hybridisation. The OD of dot blots from experimentally seeded milk with *M. bovis* increased in a linear fashion with CFU of *M. bovis* added. Using the hybridisation assay some farms were shown to be free of *M. bovis* infection. Collectively these data show that the DNA probe assay developed provides specific and sensitive means of detection of *M. bovis* DNA.

The species-specific *M. bovis* probe described in this chapter opens the way for epidemiological studies that were previously impossible. The preliminary results from

commercial dairy herds indicate that the presence of *M. bovis* in the milk from dairy cattle in north Queensland is widespread. This finding adds further weight to the suggestion that *Mycoplasma* infection of the udder could be more common in Australia and New Zealand (Hungerford 1990) than was previously thought. Further investigation should be undertaken with both bulk milk and individual animals to detect infected cattle that are shedding *M. bovis* in their milk, and to investigate its role as a possible cause of elevated SCCs.

If the probe is to be used as a routine diagnostic procedure, a non-radioactive label will be necessary. However, use of a non-radioactive label would be likely to reduce the sensitivity, as has been reported for other *Mycoplasma* probes (Hyman et al. 1987). A competent hybridisation probe for the detection of *M. bovis* could be applied in a number of ways. Because the exact role of *M. bovis* in bovine respiratory disease is not fully understood, a hybridisation probe for epidemiological studies, aetiological diagnosis of clinical infections, and pathologic studies of tissue sections using *in situ* hybridisation techniques would be a significant development.

CHAPTER 8

DEVELOPMENT OF A POLYMERASE CHAIN REACTION FOR *MYCOPLASMA BOVIS* DNA

8.1 Introduction

A sensitive and rapid laboratory diagnostic method is necessary to ensure the correct identification of the organisms and effective control of disease caused by *Mycoplasma* (Simecka et al. 1992; Kirk and Lauerman 1994). Since radionucleotide labelled DNA hybridisation is not easily adapted for routine laboratory usage, and to enhance the assay sensitivity further a PCR was developed.

Polymerase chain reaction is a powerful technique that can selectively amplify a specific segment of DNA. This method enables the detection of minute quantities of DNA, in ideal conditions a single copy of a certain target sequence, by amplifying this DNA many times using a thermostable DNA polymerase. A major advantage of using PCR is the speed at which the assay can be performed. Furthermore demonstrating presence of *M. bovis* in live animals is possible prior to a humoral response to the organism. Use of serological assays may delay recognition of the cause of the infection for up to several weeks (Razin 1994).

Polymerase chain reaction has been used for the rapid detection of a variety of pathogenic Mycoplasma, for example, *M. hyopneumoniae* (Mattsson et al. 1995), *M. agalactiae* (Tola et al. 1996), *M. pneumoniae* (Luneberg et al. 1993), *M. meleagridis* (Zhao and Yamamoto 1993), and *M. mycoides* subsp. *mycoides* (Bashiruddin et al. 1994).

A PCR for the detection of *M. bovis* using primers selected from partially sequenced *M. bovis* rRNA and flanking regions of a *Hin*dIII fragment of *M. bovis* DNA inserted in pUC19 has been reported (Hotzel et al. 1993). However, the detection limit was approximately 10^7 CFU/mL in experimentally infected milk, which is too insensitive for practical purposes. To improve the detection of *M. bovis*, the milk is trypsinised in the presence of Triton X-100 followed by a DNA-binding filtration from which the DNA is extracted and then subjected to PCR (Hotzel et al. 1996). Using this method the detection limit of *M. bovis* was improved in clinical samples to 500 CFU/mL by agarose gel electrophoresis and 50 CFU/mL after Southern hybridisation. Thus, although the use of southern blotting and radiolabelling

increased the sensitivity of the test, the advantages of speed and convenience of the PCR were lost.

This chapter describes the design of a specific and sensitive PCR assay for the detection of *M. bovis*. The main goal of this study was to develop a rapid, simple, inexpensive and robust diagnostic assay that could be eventually be used to detect and differentiate *M. bovis* from other *Mycoplasma* species and bacteria in clinical samples.

8.2 Materials and Methods

8.2.1 Selection of recombinant plasmid

The probe prepared from the *M. bovis* DNA fragment in plasmid *Mb* 1-6 (Chapter 7) only hybridised with *M. bovis* chromosomal DNA, with a sensitivity of 200 CFU/mL of target *Mycoplasma*. This probe was selected for DNA sequencing to develop primers for the *M. bovis* PCR (MB-PCR).

8.2.2 DNA sequencing

The nucleotide sequence of the purified recombinant plasmid *Mb* 1-6 DNA was determined using the Applied Biosystems Dye Prism kit following the manufacturer's recommendations. Initially, the recombinant plasmid DNA propagated in *E. coli* JM101 host strain would not yield acceptable sequencing results. Therefore the recombinant plasmid *Mb* 1-6 was transferred to the XL-1 Blue host strain, and the DNA purified as described in Section 7.2.8.2. The sequencing of recombinant plasmid was conducted in both directions. One microgram of recombinant plasmid DNA was mixed with 9.5 µL Terminator premix and 3.2 pmol of M13 forward primer (Promega; Cat. No. Q560 A) or M13 reverse primer (Promega; Cat. No. Q542A) in a final reaction volume of 20 µL which was overlaid with 40 µL of mineral oil. Plasmid pUC19 DNA (1 µg) was used as a negative control. The cycle sequencing was performed in an automated DNA thermal cycler (Bresatec; MJ research INC PTC-100™). After denaturation for five minutes at 96°C, 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C and four minutes at 60°C were performed.

8.2.3 Purification of reaction

The sequencing reaction was purified by phenol-chloroform extraction. The reaction mixture was made up to 100 µL by adding sterile distilled water. The oil was removed from the surface using a pipette. The terminators were extracted twice with 100 µL of saturated phenol:H_2O: chloroform (68:18:14). Each time the sample was vortexed then centrifuged for one minute and the aqueous phase was transferred to a clean microfuge tube. The extension products were precipitated by adding 15 µL of 2 M sodium acetate pH 4.5, and 300 µL of pure ethanol. The mixture was incubated at -70 °C for 30 minutes and then centrifuged for 15 minutes at room temperature. The supernatant was discarded and the plate washed with 70% ethanol, and then air dried. Since necessary facilities for sequencing were not available at James Cook University, the dried supernatant was sent to the DNA sequencing facility at The University of Queensland in Brisbane.

8.2.4 Primer determination

The sequence of the oligonucleotide primers to be used for PCR were selected from the results of DNA sequencing (Figure 8.1). From the DNA sequence data of recombinant plasmid *Mb* 1-6, two acceptable primers, with minimal homology to other known sequences in GenBank or EMBL DNA databases, were selected from flanking regions of this clone. These primers were synthesised by Bresatec. They were designed to amplify a target of 215 bp. The primers were dissolved in distilled water to obtain a stock solution of 400 pmol/µL, aliquoted and stored in 20 µL volumes at -20 °C.

8.2.5 Preparation of DNA template

In order to prepare the DNA template for PCR, four different methods were tested.

8.2.5.1 Boiling

Serial dilutions of *M. bovis,* from 2 x 10^6 to 100 CFU, were prepared in distilled water. They were boiled for 10 minutes and then centrifuged for three minutes at 10000 *g* (Beckman; Microfuge E™). Aliquots of 5 µL of supernatant were subjected to PCR without any further treatment of template.

8.2.5.2 Proteinase K treatment

Serial dilutions of *M. bovis* as described in Section 8.2.5.1 were treated with 100 μg/mL proteinase K at 50°C for 1 hour and then 2 μL of each dilution was subjected to PCR.

8.2.5.3 Pre-Taq treatment

Enzymatic protein digestion using thermophilic protease (Pre-Taq™; Boehringer Mannheim; Cat. No. 16964-91) was attempted (McHale et al. 1991). A 1 μL volume of crude *M. bovis* or milk sample was added to 30 μL PCR buffer containing 0.3 U Pre-*Taq*. It was incubated at 94°C for 30 minutes under 40 μL of mineral oil. The samples were centrifuged for two minutes at 10000 *g* (Beckman; Microfuge E™) and a 3-μL volume was subjected to PCR.

8.2.5.4 Phenol-chloroform extraction

The DNA samples were prepared as described in Section 7.2.1. Three microlitres of the extracted DNA sample were added to 27 μL of PCR buffer (Appendix 3.17) with 40 μL mineral oil and washed at 94°C for 30 minutes. It was centrifuged for two minutes at 10000 *g* (Beckman; Microfuge E™) and then 2 μL of supernatant was subjected to PCR reaction.

8.2.6 Optimisation of PCR

To optimise PCR parameters, the method described in the literature review Section 2.15 was used. *Taq* DNA polymerase was obtained from Bresatec, Australia (Cat. No. BTQ-1 E.017) and the reaction buffer supplied with the enzyme was used.

8.2.6.1 PCR procedure

Initially, the PCR was carried out using 25 μL volumes. Each reaction mixture consisted of 2.5 μL of 10 x reaction buffer, 2.5 μL (2 mM) of each nucleotide (dATP, dCTP, dGTP and dTTP) (Bresatec; Cat. No. K.106), 3 μL of 25 mM of $MgCl_2$, 1 μL volume containing 20 pmol of each primer (left and right), 1 μL (2 units) of *Taq* DNA polymerase and 12 μL distilled water. The PCR mix (23 μL) was dispensed into 200 μL microtubes (Bresatec). A 2-μL volume of DNA template was added to each reaction in a separate room. To prevent

evaporation of the samples, each reaction mixture was overlaid with two drops of sterile mineral oil (Bresatec; Chillout 14™). The PCR was performed in an automatic DNA thermal cycler (MJ research; PTC-100™) using the following times and temperatures. After an initial denaturation step at 94°C for five minutes, 25 cycles of a denaturation step for 60 seconds at 94°C, a primer annealing period of 60 seconds at 45°C, and a primer extension step of 60 seconds at 72°C were performed. Following this a final incubation period at 72°C for seven minutes was used.

8.2.6.2 Reagents

To determine the reaction conditions giving the greatest production of specific product with high sensitivity and minimal production of non-specific products, various concentrations of reagent were assessed. To do this various concentrations of $MgCl_2$ (1, 2, 3 and 4 mM), dNTPs (2, 1, 0.5, 0.2 and 0.1 mM), *Taq* DNA polymerase (2, 1 and 0.5 U) and primer (20, 10 and 5 pmol) were tested.

8.2.6.3 Cycling conditions

Optimisation of the annealing temperature is of paramount importance, as low annealing temperatures may result in non-specific PCR products. Initially, to prevent loss of sensitivity, a low annealing temperature of 45°C was selected. To optimise the annealing temperature a range from 45° to 60°C was evaluated. To increase the sensitivity of the test, 25, 30, 35, 38 and 40 cycles were evaluated.

8.2.7 Agarose gel electrophoresis

The PCR product was detected using agarose gel electrophoresis. Agarose gels were prepared by melting 0.48 g of agarose (1.2% agarose gel) (Progen) in 40 mL TAE buffer (Appendix 3.16) containing 0.4 µg/mL of ethidium bromide. The gels were prepared in a glass flask with heating provided by a microwave. The agarose was boiled for a few seconds to dissolve all of the crystals, cooled slightly so as not to damage the gel tray, and poured into the gel mould. After 20 minutes, when the gel was completely set, it was used. The PCR product (5-10 µL) was then mixed with 1 µL TAE loading buffer (Appendix 3.3), loaded into the gel and electrophoresed for 45 minutes at 100 V in 1 x TAE buffer. DNA molecular size markers 1 kb

DNA ladder (Gibco BRL; Cat. No. 15615-016) were run with a positive control using recombinant probe *Mb* 1-6 and a negative control of PCR buffer without template. The DNA was then visualised using ultraviolet light.

8.2.8 Specificity of the MB-PCR

Twenty nanograms of chromosomal DNA from each of two *M. bovis* strains (Section 7.2.1) was subjected to the MB-PCR. A 100 ng amount of DNA from each heterologous bovine *Mycoplasma* species, pUC19 plasmid and other bacteria, as listed in Table 7.1 of Chapter 7, were tested using the PCR to determine the specificity of the test.

8.2.9 Validity of MB-PCR

The validity of the PCR assay was determined by testing the DNA extracted from the 10 bulk milk samples and also the DNA from milk samples from individual cows (n:127) as used in the hybridisation assay (Section 7.2.14).

8.2.10 Sensitivity of the MB-PCR

To check the sensitivity of the MB-PCR the uninfected milk sample (Sample No. 12) which was negative for *Mycoplasma* by dot blot hybridisation and PCR, was seeded with a serial dilutions of *M. bovis* ranging from 10^9 to 20-30 CFU/mL. The DNA from each seeded sample was extracted with phenol-chloroform as described in Section 7.2.5. Precipitated DNA was dissolved in 30 µL of PCR buffer (pH 8.3) (Appendix 3.17), treated at 94°C under 40 µL mineral oil for 30 minutes and centrifuged at 10000 *g* (Beckman; Microfuge E™) for two minutes. A 5 µL aliquot of each sample was subjected to MB-PCR.

8.3 Results

8.3.1 DNA sequencing and primer determination for PCR

The use of plasmid DNA grown in the host strain XL-1 Blue for sequencing gave excellent results (Figure 8.1). The results of both directions of recombinant plasmid were the same.

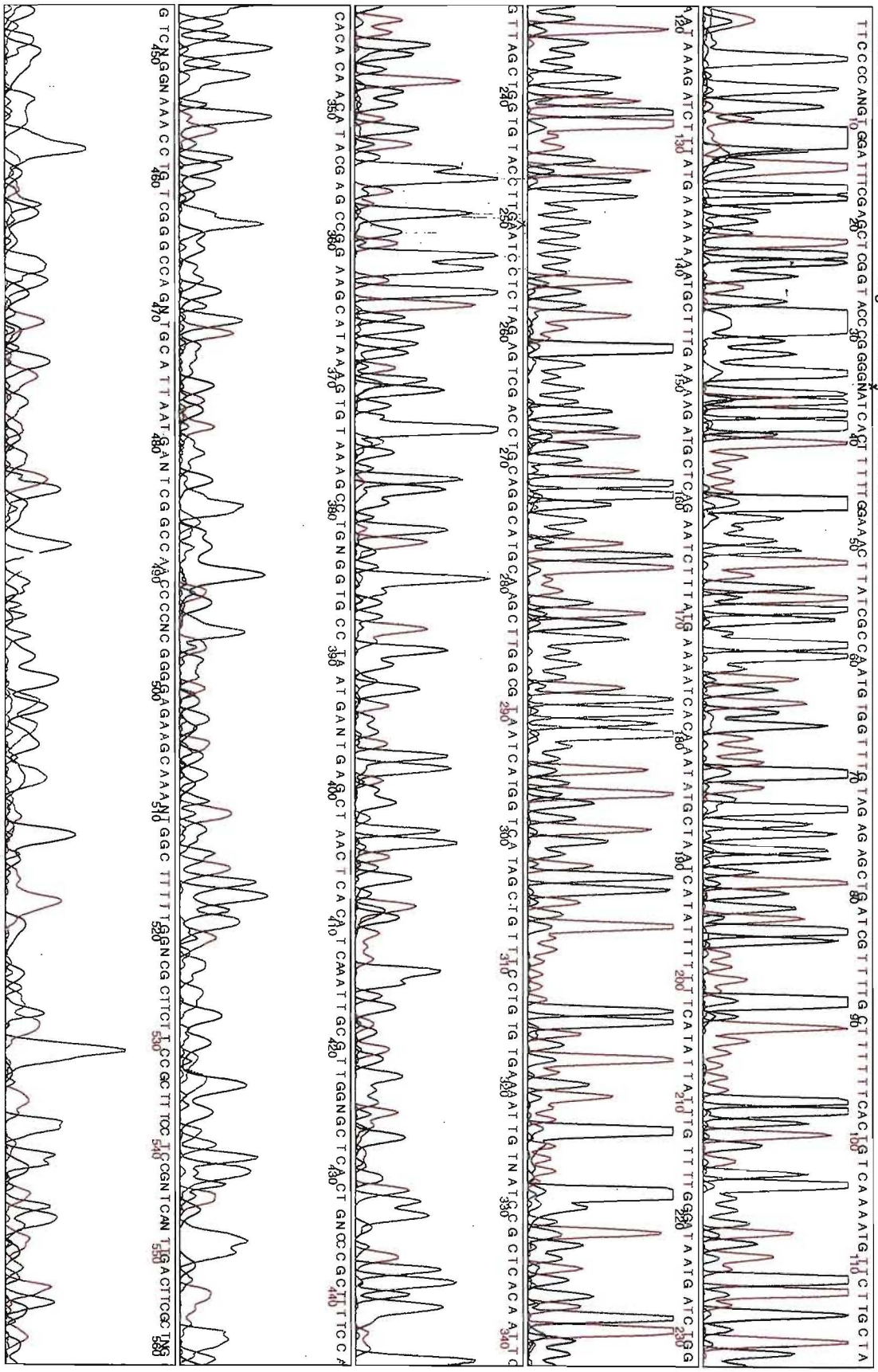

Figure 8.1 Nucleotide sequence data generated from cycle sequencing of recombinant plasmid 1-6 using M 13 forward primer.

A computer search for sequence similarity was conducted. Based on analysis of the DNA sequencing data of *Mb* 1-6, the clone revealed a 215 nt fragment which showed little resemblance to any sequence contained in GenBank or the EMBL DNA database. Primer *Mb* 1-6s had no homology with known sequences in GenBank and primer *Mb* 1-6 showed 100% score homology with the vspA sequence of *M. bovis* (strain PG-45) (Lysyansky et al. 1996). The primer sequences and size of primers derived from clone *Mb* 1-6 are as follows:

Primer	Sequence	No. of Bases	G + C%	Tm °C
Mb 1-6	5' AAGGTACACCAGCTAACCCAG 3'	21	52.38	54.2
Mb 1-6s	5' GATCACTTTTTGGAAACTTAT 3'	21	28.57	44.5

8.3.2 Preparation of DNA template

8.3.2.1 Boiling method

With template prepared by the boiling method 50 and 100 CFU of *M. bovis* were detectable by PCR but high template concentrations inhibited PCR products (Figure 8.2).

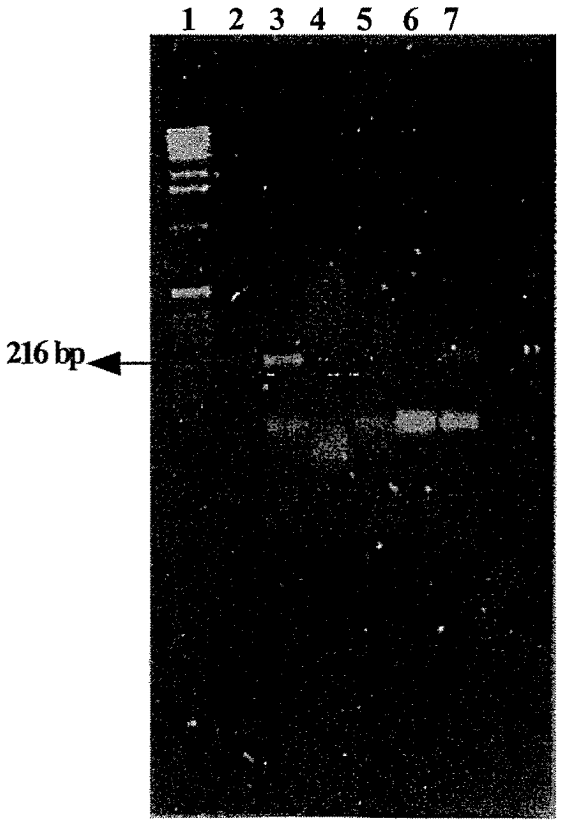

Figure 8.2 Agarose gel electrophoresis of polymerase chain reaction products obtained by using the boiling method of sample preparation.
Lane 1: MWM (BRL 1 Kb ladder)
Lane 2: 50 CFU *M. bovis*
Lane 3: 100 CFU *M. bovis*
Lane 4: 10^3 CFU *M. bovis*
Lane 5: 10^4 CFU *M. bovis*
Lane 6: Negative control (no template)
Lane 7: Positive control (recombinant plasmid (1-6))

8.3.2.2 Proteinase K treatment

No amplified region was observed using proteinase K treatment (Section 8.2.5.2) with all of the reactions completely inhibited (Figure 8.3).

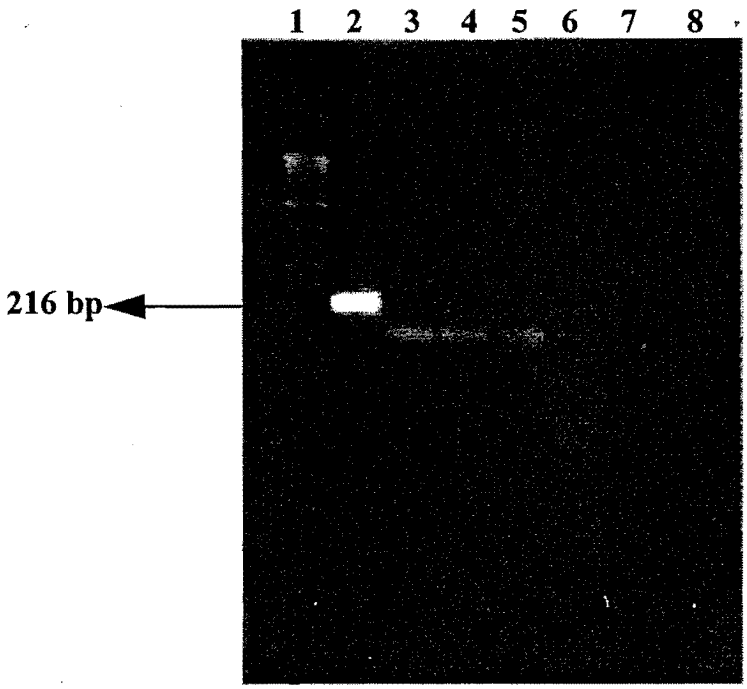

Figure 8.3 Agarose gel electrophoresis of polymerase chain reaction products obtained by proteinase K treatment of samples.
Lane 1: MWM (BRL 1 Kb ladder)
Lane 2: Positive control (recombinant plasmid (1-6))
Lane 3: Negative control (no template)
Lane 4: 50 CFU *M. bovis*
Lane 5: 100 CFU *M. bovis*
Lane 4: 10^3 CFU *M. bovis*
Lane 5: 10^4 CFU *M. bovis*
Lane 6: 10^5 CFU *M. bovis*
Lane 7: 10^6 CFU *M. bovis*
Lane 8: 2 x 10^6 CFU *M. bovis*

8.3.2.3 Pre-Taq treatment

While the first two methods gave poorly reproducible and unsatisfactory results, the enzymatic procedure using Pre-*Taq* led to a significant improvement in sensitivity (Figure 8.4). However, the result was unstable due to the effect of storage on the enzyme. Prolonged storage was found to dramatically reduce *Taq* polymerase activity.

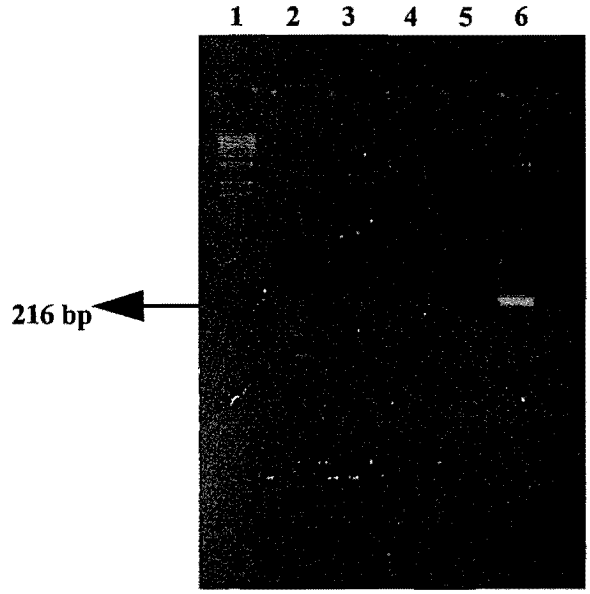

Figure 8.4 Agarose gel electrophoresis of PCR products obtained by pre-*Taq* treatment of milk samples.
Lane 1: MWM (BRL 1 kb ladder)
Lane 2: Infected milk with 10^4 CFU *M. bovis*
Lane 3: Infected milk with 10^3 CFU *M. bovis*
Lane 4: Infected milk with 500 CFU *M. bovis*
Lane 5: Negative control (no template)
Lane 6: Positive control (recombinant plasmid (1-6))

8.3.2.4 Phenol-chloroform extraction

The template prepared by phenol-chloroform extraction following pre-treatment with PCR buffer yielded an optimal results (Figures 8.5 and 8.6). It was concluded that the extraction of DNA, and its pre-treatment with PCR buffer, was the best method of template preparation for this PCR.

8.3.3 $MgCl_2$ concentration

The optimal $MgCl_2$ concentration, from the ranges of 0.5, 1, 1.5, 2, 3 and 4 mM tested, was 3.0 mM. The effect of $MgCl_2$ concentration on PCR product is shown in Figure 8.5.

8.3.4 Cycle number

Initially a cycle number of 25 was chosen, which generated a specific product of the correct size. To try to increase the amount of product generated and so increase sensitivity, the cycle number was increased to 38 resulting in increased sensitivity. Some of these results are shown in Figures 8.5 and 8.6.

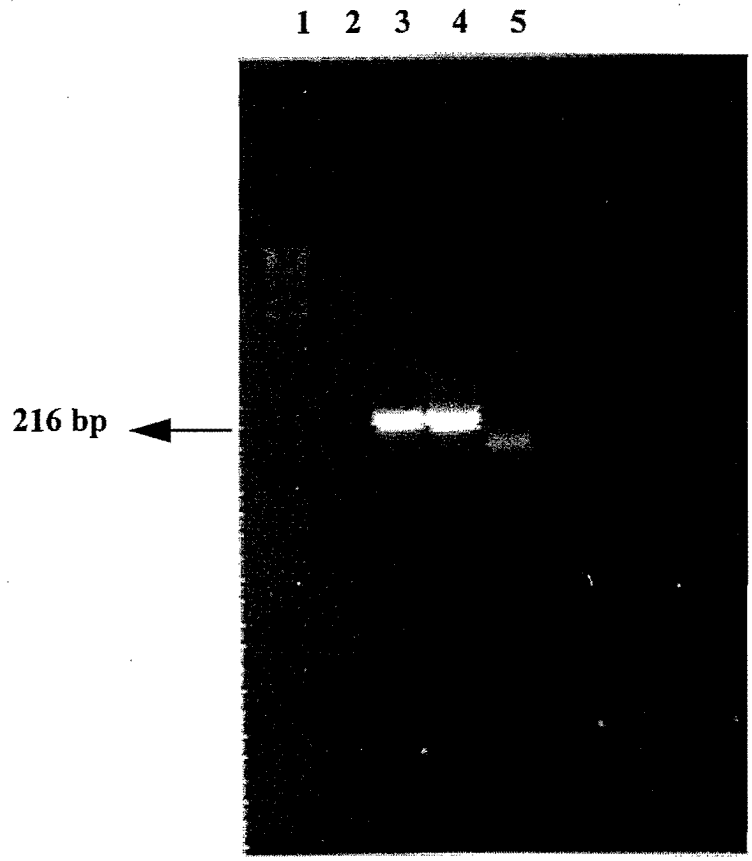

Figure 8.5 Effect of altering $MgCl_2$ concentrations using recombinant plasmid DNA (1-6) as template and 25 cycle number for *Mycoplasma bovis* polymerase chain reaction.
Lane 1: MWM (BRL 1 kb ladder)
Lane 2: PCR using 1 mM $MgCl_2$
Lane 3: PCR using 2 mM $MgCl_2$
Lane 4: PCR using 3 mM $MgCl_2$
Lane 5: Negative control (no template)

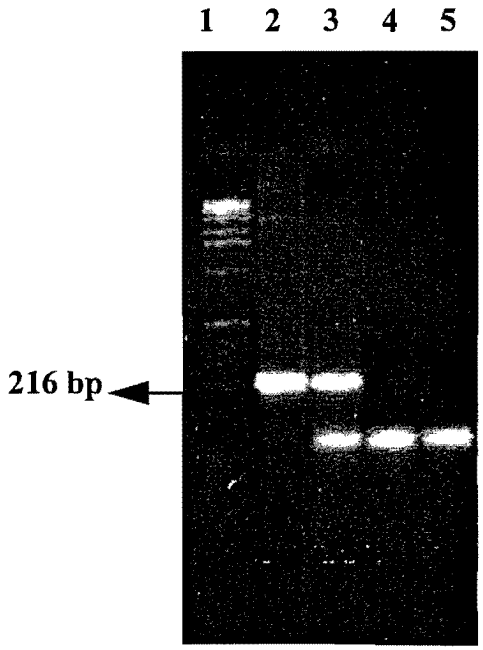

Figure 8.6 Effect of increasing cycle number to 38.
Lane 1: MWM (BRL 1 kb ladder)
Lane 2: PCR using recombinant plasmid DNA (1-6) as target
Lane 3: PCR using *M. bovis* DNA as a target
Lane 4 & 5: Negative control (no template)

8.3.5 Annealing temperature

The initial annealing temperature of 45°C for one minute resulted in a non-specific product (Figure 8.7). When the annealing temperature was increased to 55°C, this non-specific product did not occur (Figure 8.8).

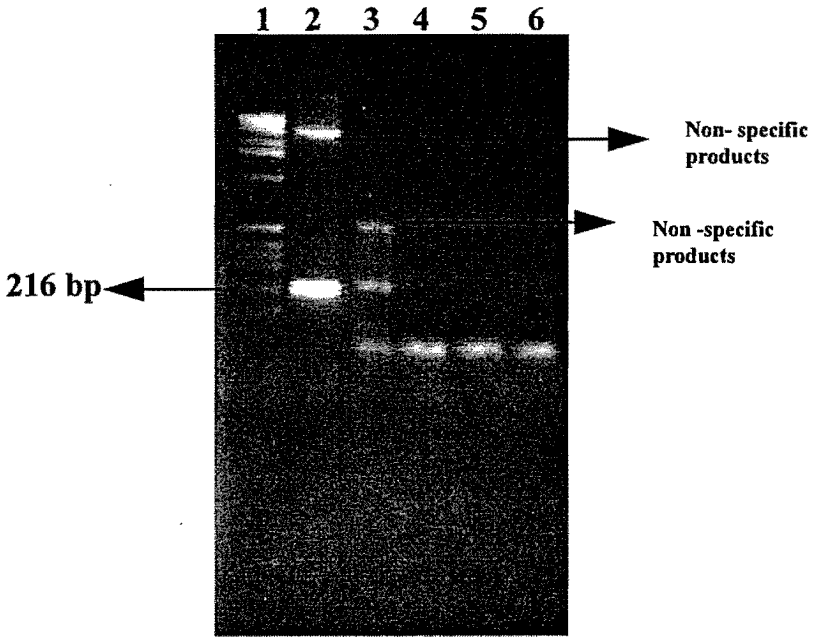

Figure 8.7 Non-specific polymerase chain reaction products produced at low annealing temperature (45°C).
Lane 1: MWM (BRL 1 kb ladder)
Lane 2: Positive control (recombinant plasmid (1-6))
Lane 3: *M. bovis* DNA
Lane 4, 5 & 6: Negative control (no template)

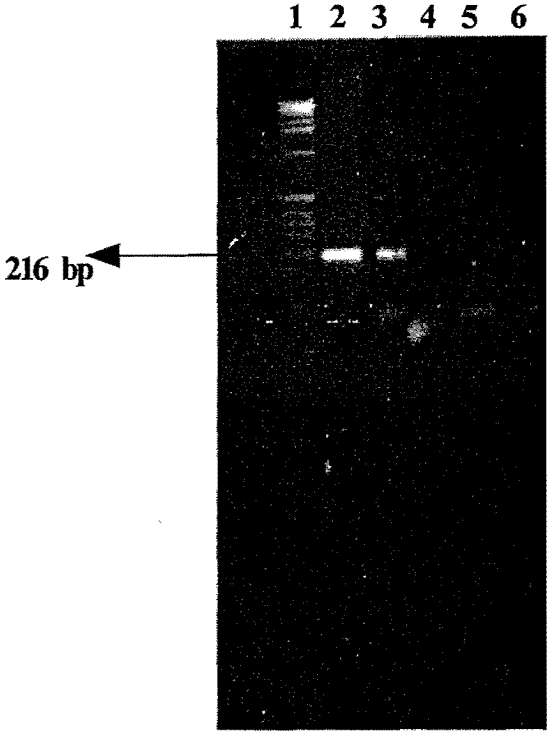

Figure 8.8 Effect of increasing the annealing temperature to remove non-specific product (as seen in Figure 8.7).
Lane 1: MWM (BRL 1 kb ladder)
Lane 2: PCR using plasmid DNA as target (positive control)
Lane 3: PCR using *M. bovis* DNA as target with annealing temperature of 55 °C
Lane 4, 5 & 6: Negative control (no template)

8.3.6 Primer concentration

Primer concentrations of 20 pmol and 10 pmol per reaction were tested. No loss in activity was seen when the lower primer concentration was used, so use of this amount was adopted.

8.3.7 Optimisation of the PCR

To gain maximum sensitivity and specificity all parameters of the PCR were optimised. After optimisation the PCR was carried out in 25 µL reaction volumes containing 2.5 µL 10 x PCR buffer; 10 pmol of each primer; 0.1 mM of each dNTP; 3 mM $MgCl_2$; and 1 Unit *Taq* DNA polymerase. The reaction was performed using 38 cycles and the optimal temperature profile of a cycle for amplification of a 215-bp *M. bovis* fragment after a denaturation step for five minutes at 94 °C was a denaturation step at 94 °C for 60 seconds, primer annealing at 55 °C for 60 seconds, and primer extension at 72 °C for 60 seconds. The final incubation was at 72 °C for seven minutes.

8.3.8 Specificity of MB-PCR

Samples of 20 ng of DNA prepared from two strains of *M. bovis* were amplified, and yielded a 215-nt product as expected. Samples of DNA (100 ng) from eight other bovine *Mycoplasma* species, and also other bacteria as listed in Table 7.1 (Chapter 7) were tested, and no product was observed (Figure 8.9).

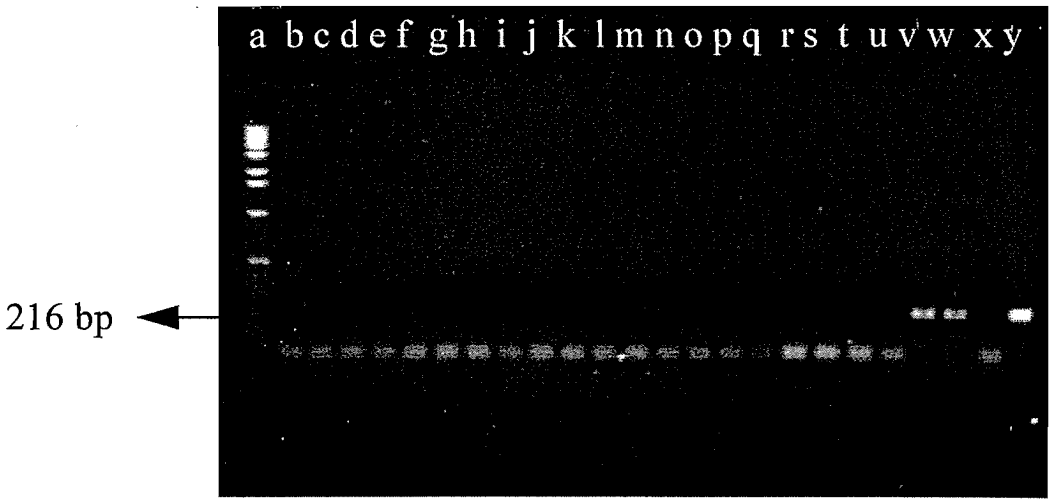

Figure 8.9 Agarose gel electrophoresis showing the *Mycoplasma bovis* polymerase chain reaction products using genomic DNA of various species as template.
Lane a: MWM (BRL 1 Kb ladder)
Lane b: *Staphylococcus aureus*
Lane c: *Staphylococcus* spp.
Lane d: *Streptococcus uberis*
Lane e: *Streptococcus agalactiae*
Lane f: *Corynebacterium bovis*
Lane g: *Serratia marcescens*
Lane h: *Enterobacter cloacae*
Lane i: *Klebsiella pneumoniae*
Lane j: *Pseudomonas aeruginosa*
Lane k: *Pasteurella* spp.
Lane l: *Brucella abortus*
Lane m: *Yersinia enterocolitica*
Lane n: *Mycoplasma bovirhinis*
Lane o: *Mycoplasma group 7*
Lane p: *Mycoplasma ovipneumoniae*
Lane q: *Mycoplasma arginini*
Lane r: *Mycoplasma dispar*
Lane s: *Mycoplasma agalactiae*
Lane t: *Mycoplasma bovigenitalium*
Lane u: *Mycoplasma bovirhinis*
Lane v: *Mycoplasma bovis* (type strain)
Lane w: *Mycoplasma bovis* (Oonoonba strain)
Lane x: No template (negative control)
Lane y: Recombinant plasmid (1-6) (positive control)

8.3.9 Validity of MB-PCR

The PCR assay was validated by using the DNA extracted from the bulk milk samples and composite milk samples from individual cows, as used in the hybridisation assay (Section 7.2.14). The PCR assay confirmed the hybridisation results. Nine of 10 bulk milk samples were found to be positive for *M. bovis* by PCR. At herd level, the PCR assay revealed that 66.9% (85 out of 127) of cows were secreting *M. bovis*. The PCR assay results on bulk milk samples are demonstrated in Figure 8.10, and some results from composite milk from individual cows in Figures 8.11, 8.12 and 8.13. No background or inhibition of PCR associated with protein, fats or ions present in the milk were encountered in detection of *M. bovis* by PCR when the template DNA was prepared using phenol-chloroform extraction following pre-treatment with PCR buffer.

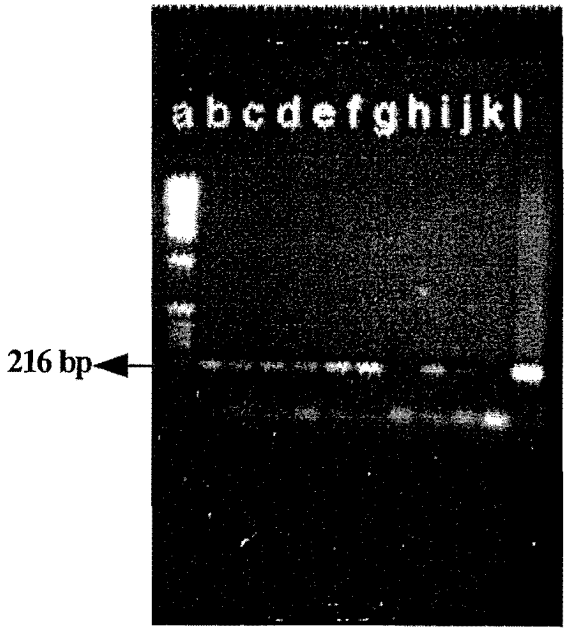

Figure 8.10 Agarose electrophoresis gel showing the reaction products from the optimised PCR amplification carried out with 9 bulk milk samples.
Lane a: MWM (BRL 1 kb ladder)
Lanes b-j: Bulk milk samples
Lane k: Negative control (no template)
Lane l: Positive control (recombinant plasmid (1-6))

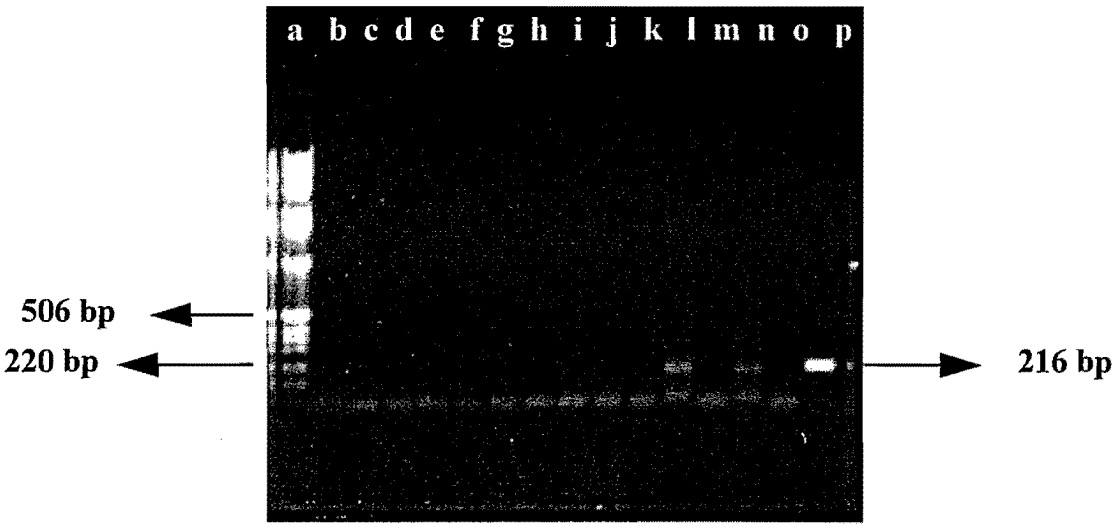

Figure 8.11 Agarose electrophoresis gel showing the reaction products from the optimised polymerase chain reaction amplification carried out with 13 composite milk samples from individual cows from the Atherton Tableland, north Queensland.

Lane a: MWM (BRL 1 kb ladder)
Lane b-n: Milk samples from individual cows
Lane o: Negative control (no template)
Lane p: Positive control (recombinant plasmid (1-6))

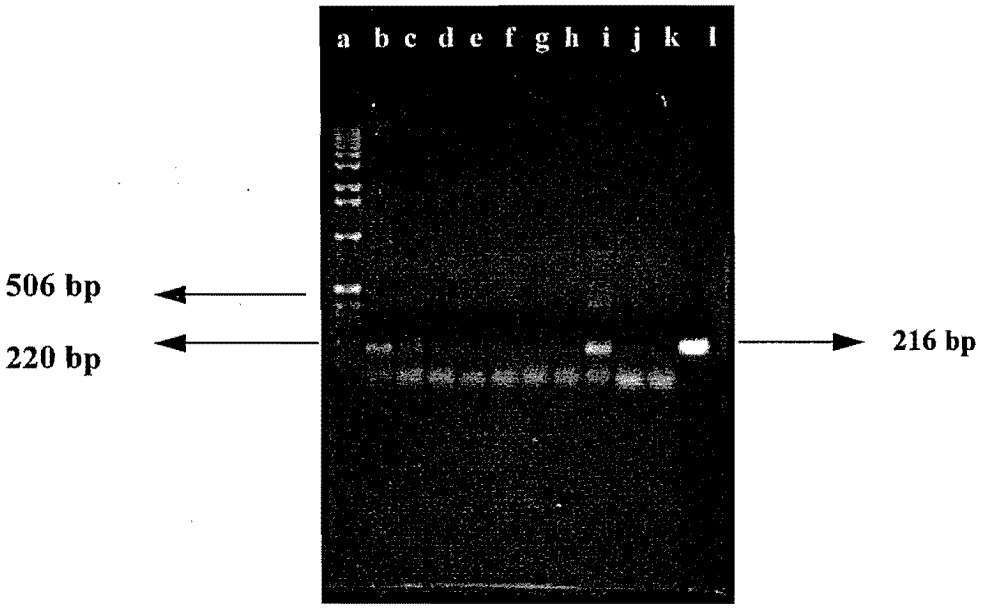

Figure 8.12 Agarose electrophoresis gel showing the reaction products from the optimised polymerase chain reaction amplification carried out with nine quarter milk samples from individual cows from the Atherton Tableland, north Queensland.
Lane a: MWM (BRL 1 kb ladder)
Lane b-j: Quarter milk samples from individual cows
Lane k: Negative control (no template)
Lane l: Positive control (recombinant plasmid (1-6))

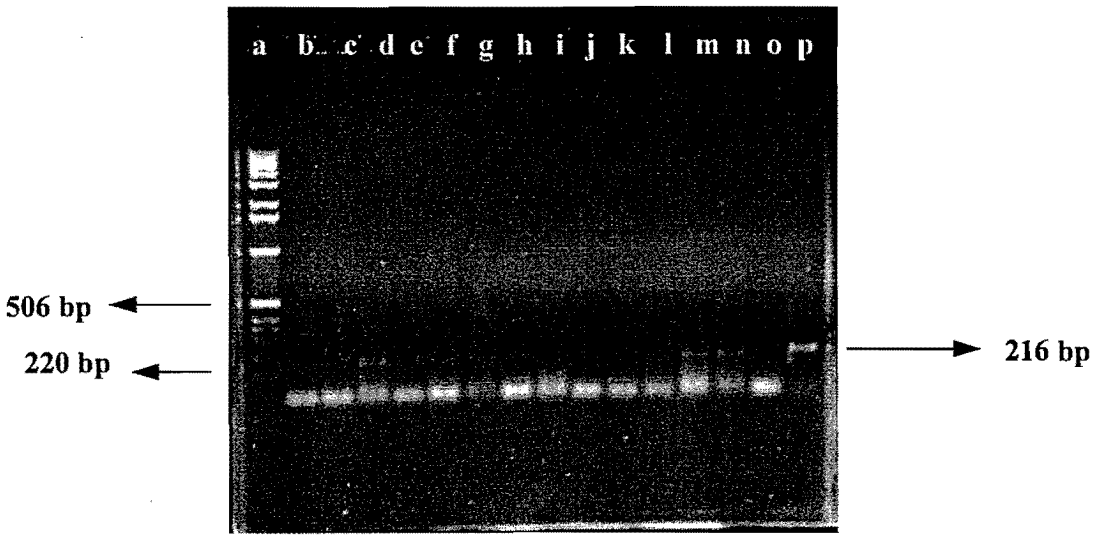

Figure 8.13 Agarose electrophoresis gel showing the reaction products from the optimised polymerase chain reaction amplification carried out using 13 quarter milk samples from individual cows from the Atherton Tableland, north Queensland.
Lane a: MWM (BRL 1 kb ladder)
Lane b-n: Quarter milk samples from individual cows
Lane o: Negative control (no template)
Lane p: Positive control (recombinant plasmid (1-6))

8.3.10 Sensitivity of MB-PCR

The sensitivity of the MB-PCR was approximately 20 *M. bovis* CFU/mL of homologous DNA in the milk as determined by 1.5% agarose gel electrophoresis (Figure 8.14). As can be seen in Figure 8.14 the amount of PCR product from experimentally infected milk samples increased with increasing numbers of *M. bovis* in the milk. The PCR is 10-fold more sensitive than hybridisation.

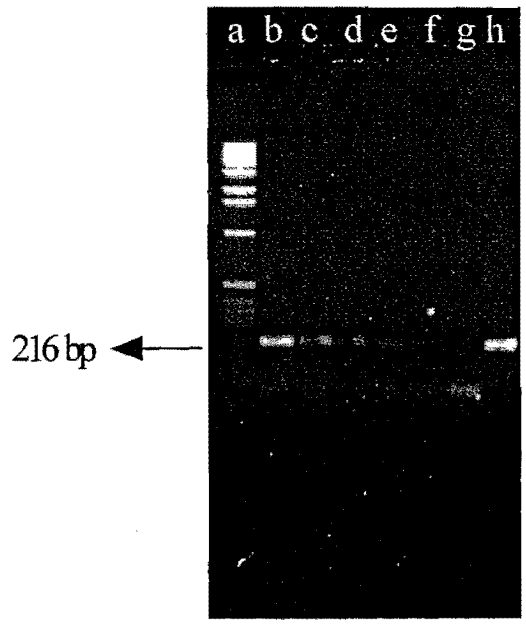

Figure 8.14 Agarose gel electrophoresis showing the polymerase chain reaction amplification reaction products using various dilutions of *Mycoplasma bovis* genomic DNA in experimentally infected milk. The DNA was extract from milk containing 10^9 (Lane b), 10^5 (Lane c), 10^3 (Lane d), 300 (Lane e) and 20-30 (Lane f) CFU *M. bovis*/mL. Lane a contains the MWM (BRL 1 kb ladder), Lane g has no template and Lane h used the recombinant pUC19 plasmid DNA as positive control.

8.4 Discussion

The methods currently used to diagnosis *M. bovis* infection have limitations regarding sensitivity, time and cost (Simecka et al. 1992; Kirk and Lauerman 1994). Polymerase chain reaction has been used for the rapid detection of a variety of pathogenic *Mycoplasma*, and therefore is a promising candidate to replace current diagnostic methods. Economic and animal health requirements are driving the search for ways to control *Mycoplasma* disease (Ross 1992). Thus a main goal of this study was to design a rapid, sensitive and specific PCR assay that could eventually be used to detect and differentiate *M. bovis* from other *Mycoplasma* species and other bacterial species in clinical samples.

The first step in development of any PCR assay is to identify and obtain a target sequence and establish a PCR protocol (Persing 1993). This involves design of *M. bovis*-specific primers, optimisation of the parameters of the reaction to provide the greatest sensitivity and specificity, and the design of a protocol requiring minimum sample manipulation.

Due to the limited sequence data available for *M. bovis*, the primers were designed from the DNA probe described in Chapter 7, which could discriminate *M. bovis* from all other *Mycoplasma* species tested. Two oligonucleotides complementary to two sequences flanking a 215-nt fragment from clone *Mb* 1-6 were selected. The primers were chosen based on compatible high primer annealing temperatures, low self complementarity and an absence of

inter-primer complementarity, especially at the 3' end. Although the OLIGO program analysis showed that primers (*Mb* 1-6) and *(Mb* 1-6s) formed some hairpin loop structures and primer dimers, these were not expressed at the elevated annealing temperature of 55 °C chosen in the PCR protocol. Similarly, while there was some self-complementarity between the two primers, they did not form primer-dimers at the annealing temperatures employed.

The prerequisite of the PCR assay was that it should specifically detect *M. bovis* but not cross-react with other *Mycoplasma* species and bacteria which are likely to be found in clinical samples. Initially, the selected primers were screened against all sequences in GenBank and EMBL databases. While one of the primers had no homology with sequences of microorganisms in GenBank, the other showed 100 % homology with the vspA gene of *M. bovis* (strain PG-45) (Lysnyansky et al. 1996). Having selected the primers, the PCR was optimised to provide maximum sensitivity and specificity.

The specificity of the primers was very high. Even high concentrations (100 ng) of heterologous bovine *Mycoplasmas* species DNA and DNA from other bacteria likely to be found in clinical samples were not amplified. An important observation was that DNA from *M. agalactiae,* a closely related species (Askaa and Erno 1976), was not amplified.

Removal of interfering proteins is of paramount important for the PCR detection of *Mycoplasma* in biological samples (Hotzel et al. 1996). Several protocols for preparation of template for PCR amplification were tested. It was found that organic solvent extraction using phenol-chloroform gave the most reproducible results. Although this method may ensure removal of inhibitors, the residual organic reagents used for the purification of the nucleic acid may themselves inhibit the reaction (Rossen et al. 1992). They may act by binding to target DNA thus preventing access by primers or polymerases, or interfere with enzyme activity by binding to *Taq* DNA polymerase. They may also upset the buffer balance. Other potential inhibitors include proteinases (plasmin) (Powell et al. 1994), DNases or RNases that inhibit the reaction by degradation of either the enzyme or target DNA (Persing 1993).

To inactivate these potential inhibitors of the MB-PCR, and to increase sensitivity after phenol-chloroform extraction, the DNA samples were first treated with PCR buffer. This substantially increased the sensitivity of the assay for the detection of *M. bovis* DNA from bulk milk samples.

Since the standard DNA purification procedure using phenol-chloroform extraction involved excessive sample manipulation, alternative purification techniques were also tested. While Pre *Taq* yielded good results, the enzyme was unstable, as its activity was dramatically reduced on storage and it was not used in the final protocol.

Interference by proteins, fats and ions present in the milk may cause false positive and false negative results for PCR detection of *M. bovis* in milk (Hotzel et al. 1993). In this study no background problems were encountered after the DNA template was extracted.

One well-known problem with PCR is false positive results due to contamination of test material, most often with amplicons from preceding reactions. In this study no problems with false positive results were detected. Strict adherence to the physical separation of pre- and post-PCR work areas was sufficient to prevent such contamination.

One potential application of the MB-PCR developed in this study was for the detection of *M. bovis* in milk. The minimum amount of homologous DNA that could be detected by the MB-PCR was 20 CFU, which makes it 10 times more sensitive than dot blot hybridisation. To validate this assay, bulk milk and composite milk from individual cows from farms on the Atherton Tableland were tested. The PCR confirmed the results from the bulk milk hybridisation assay and, for detection of *M. bovis* from individual cows was more sensitive (66.9%) than the hybridisation assay (59%). This result suggests a high prevalence of *M. bovis* infection in the udder of dairy cattle in north Queensland. The assay has proven to be suitable for detection of *Mycoplasma* in clinical samples, is much faster than existing methods and is adaptable to automation for large scale screening. It is more sensitive and successful than techniques currently used for the detection of *Mycoplasma* in clinical samples and should prove invaluable in the investigation of the pathogenesis of *Mycoplasma* infections.

Large scale screening of milk is now possible for the first time through the use of this highly sensitive and specific PCR assay. Further investigation must be undertaken on bulk milk samples and on individual farms to detect infected cattle that are shedding *M. bovis* in their milk, and to investigate their role as a possible cause of elevated SCCs. This has important implications in milk quality assurance programs and herd management.

CHAPTER 9

PRELIMINARY STUDIES ON THE PREVALENCE OF *MYCOPLASMA BOVIS* MASTITIS IN DAIRY CATTLE

9.1 Introduction

Mastitis, or inflammation of the mammary gland, is one of the most complex and costly diseases of dairy cattle worldwide (Eberhart et al. 1987). Mastitis is known to have a multitude of effects on the quantity, quality and processing properties of the milk produced. It results from the introduction and multiplication of pathogenic microorganisms in the mammary gland which leads to reduced milk production, compositional changes and elevated SCC. The primary source of most infections is thought to be through subclinical or chronically infected glands that are more common in the older cows within each herd. At least 50% of Australian dairy herds suffer subclinical gland infections. The most recent information obtainable estimates that mastitis costs the Australian dairy industry more than $60 million per year (Munro et al. 1984). Losses to the milk producer arise from decreased milk production, cost of treatment and wastage of cows. To the processor, losses occur from faulty production runs in the manufacture of cultured products, inflated processing costs, difficulty in marketing inferior products, and then to the consumer from increased expense for inferior quality products (Giesecke 1979).

Subclinical infection may go undetected without the application of diagnostic tests to the milk. It may be prolonged or proceed rapidly to clinical mastitis, where external signs of disease such as swelling and hardness of the affected glands or clots or discolouration of the secretion are present. The subclinically infected quarter has a lowered milk yield, altered milk composition and is a reservoir of infection within the herd.

There is no easy, simple approach to mastitis control. In a mastitis control program, it is much more appropriate to attempt to prevent all types of infection rather than to concentrate on eradication of only one or two types of infection (McDonald 1984).

Determining the aetiology of infection without laboratory testing of milk samples is not possible. Although many clinical cases of mastitis that are due to infection are negative on bacteriological culture of the mammary gland secretion (Bulletine 1987), research on the

aetiological agents generally has not considered the *Mycoplasma*. Unfortunately culture, suggested as the gold standard (Jasper 1981), has poor sensitivity for the detection of *Mycoplasma* in the milk. Therefore alternative assays, such as a DNA probe or a PCR, for the detection of *M. bovis* in milk have been suggested (Simecka et al. 1992).

The preliminary results of studies using a DNA probe and the MB-PCR on bulk milk samples (Chapters 7 and 8) indicated that *Mycoplasma* mastitis may be a major udder health problem in dairy cattle in north Queensland. Although the isolation of *M. bovis* associated with mastitis was first reported by Cottew (1970), since then there has been no report on the isolation or recognition of *M. bovis* in dairy cattle in Australia.

In this chapter, some preliminary background data on the prevalence of *M. bovis* infection in dairy cattle obtained through the application of the MB-PCR are reported. To do this, the presence of *M. bovis* in bulk milk samples from herds in north Queensland and Victoria, in composite milk samples from a single herd on the Atherton Tableland, and in milk from cows with mastitis refractory to treatment was investigated. The persistence of *M. bovis* infection, its association with SCC and serology and the potential role of the respiratory tract in the spread of *M. bovis* and its relationship to mastitis were investigated.

These studies should provide a better understanding of *M. bovis* as a disease determinant and its distribution among dairy herds to assist with improving udder health. Through improved udder health, profitability to the producer should be increased and the quality of milk would be assured.

9.2 Material and Methods

9.2.1 Distribution of *M. bovis* within region – bulk milk sampling

A total of 186 and 165 bulk milk samples from north Queensland and Victoria respectively was obtained from milk factories following routine somatic cell counting. The samples were received on ice without preservative.

9.2.2 Distribution of *M. bovis* within herds – composite milk samples

A single collection of 92 composite milk samples from individual cows was made from a single herd (Farm 1) on the Atherton Tableland. Additionally, composite milk samples from individual cows with high and low SCC on Farm 1 and another farm (Farm 3) were obtained at monthly intervals for varying periods up to six months. The samples were collected by the local veterinary practitioner or agriculture officers from the Queensland Department of Primary Industries on the Atherton Tableland. They were stored on ice and transported to the laboratory.

9.2.3 Association of *M. bovis* with persistent clinical and subclinical mastitis – Quarter milk samples

To investigate the association of *M. bovis* with mastitis refractory to treatment, individual quarter milk samples from cows with a persistently high SCC from six different herds on the Atherton Tableland were obtained from the Oonoonba Veterinary Laboratory following culture for major mastitis pathogen (MMP) (*Streptococcus agalactiae, Streptococcus dysgalactiae, Staphylococcus aureus*) and non-MMP (environmental pathogens). The samples were packaged on ice and transported to the laboratory. The observation of abnormal milk (flakes, clots, or watery secretion) or blind quarters was recorded. They were tested in the Department of Microbiology and Immunology at James Cook University by PCR for *M. bovis* (as described in Chapter 8). No SCC data was obtained for these cows at the time the milk samples were collected.

9.2.4 Somatic cell counts

All study farms belonged to the Queensland Dairy Herd Improvement scheme and thus reliable SCC data for both bulk milk and composite milk was obtained from the Queensland Dairy Herd Improvement Centre (QDHIC) Wacol in Brisbane. Retrospective SCC data was obtained from QDHIC to enable the temporal association of *M. bovis* infection with SCC to be determined.

9.2.5 Herd visits

To obtain a better understanding of the condition of the study herds, a visit to two of the farms on the Atherton Tableland was undertaken. Each herd was pasture fed and milked in herringbone milking sheds.

9.2.5.1 Clinical observations

On Farm 1, general management was below average, and cows were fed during milking with molasses. In contrast, Farm 2 had superior management using advanced computerised milking technology, cows were fed 5-6 kg/cow of concentrate during milking. The average production on Farms 1 and 2 was 9.0 and 16 litres per cow per day respectively.

On Farm 1, the pre-milking hygiene was poor and most of the cows observed had loose faeces, probably as a result of feeding with molasses. The dirty teats were washed with water that reducing the pre-milking sanitation. On Farm 2, the pre-milking routine involved a 'dry-wipe' with a disposable paper towel of all but the most dirty teats, which were washed before dry-wiping.

On both farms, post-milking teat disinfection was achieved by using an iodophore spray. Dry cow therapy was administrated to all cows at the end of lactation by using a long-acting cloxacillin intra-mammary preparation. Clinical mastitis treatment consisted of milking-out the infected quarter then injecting a cloxacillin intra-mammary preparation into the quarter every 24 hours for 3-4 days.

Quarter milk samples, blood samples and nasal swabs were collected from cows and calves, where available, on both farms.

9.2.5.2 Milk samples

Quarter milk samples were collected from 14 and 15 cows, selected for high and low SCC determined from the previous herd test, from Farm 1 and Farm 2 respectively. At collection, the udder was cleaned of gross contamination by wiping with a dry paper towel and 70% ethanol was sprayed onto the teat. The first few millilitres of fore-milk were discarded and

then approximately 5 mL milk was collected from each quarter in the order right hind, right fore, left fore and left hind into sterile 30 mL universal bottles for microbiological testing. The samples were stored on ice and transported to the laboratory.

9.2.5.3 Blood samples

To determine the antibody titre to *M. bovis,* blood samples were taken from selected cows. The serum was separated and then tested by indirect ELISA (Chapter 4) and blocking ELISA (Chapter 6).

9.2.5.4 Nasal swab samples

Cotton nasal swabs were collected from 15 apparently normal cows on Farm 2 and three calves from Farm 1. Each was restrained manually and the swab inserted deep into the nasal cavity and rotated several times. Each cow was restrained in a cattle crush and the head immobilised using nose pliers. Care was taken to insert the swab deep into the nasal cavity, avoiding contact with the pliers to prevent cross infection between samples. Nasal swabs were not collected from cows on Farm 1 as there were no suitable restraining facilities available. The swab stick was broken off into a sterile tube containing 2 mL MB.

9.2.6 *Mycoplasma* culture

The quarter milk samples were cultured onto MA using the dilution method. For direct culture, 50 µL of milk was spotted onto a MA plate. Additionally, 300 µL of each milk sample was inoculated in MB and cultured as described in Chapter 4.

9.2.7 Bacteriological culture

A total of 202 quarter milk samples from 52 cows (six quarters were blind) detected through the DHIC as having persistently high composite-milk SCC were cultured at the Oonoonba Veterinary laboratories for bacterial pathogens and also tested by PCR at James Cook University Department of Microbiology and Immunology for *M. bovis*.

9.2.8 DNA extraction from nasal swabs and milk

The tubes containing the nasal swabs were vortexed and then the swabs discarded. Aliquots of 0.3 mL of each nasal swab suspension, or 0.3 mL volume of each milk sample were treated with an equal volume of NTE buffer (pH 7.4) containing SDS and proteinase K at a final concentration of 0.5% and 100 μg/mL respectively. The DNA was purified with phenol chloroform as described in Chapter 7.

9.2.9 Statistical analysis

The data for BMSCC and *M. bovis* detection by PCR for both the Atherton Tableland and Victoria were analysed by the Sigma Stat program (Jandel Scientific). The Mann-Whitney Rank Sum Test was used for comparing the BMSCC between the regions and the Chi-square test was used to find the relationship between *M. bovis* detected by PCR and SCC results for each region.

9.3 Results

9.3.1 Bulk milk samples

The summary data of the MB-PCR and SSC on the bulk milk samples from the Atherton Tableland and from Victoria are given in Table 9.1 and Table 9.2 respectively (the raw data are shown in Appendix 4.1 and 4.2). Based on published guidelines (O'Rourke and Blowey 1992) for the interpretation of SCC in bulk milk as an estimate of the level of the mastitis problem in a herd, five groups were recognised: slight $<250 \times 10^3$, average $250\text{-}500 \times 10^3$, above average $500\text{-}750 \times 10^3$, bad $750\text{-}1000 \times 10^3$ and severe $>1000 \times 10^3$. About 60% and 72% of bulk milk samples from north Queensland and Victoria, respectively, had SCC greater than 250×10^3. In the north Queensland milk samples, there was an association between *M. bovis* infection and increasing SCC (Table 9.1) and therefore with the level of mastitis. For the Victorian bulk milk samples, the same pattern of association was present (Table 9.2).

Using the Mann-Whitney Rank Sum Test, the differences in the median values between the SCC values from Atherton Tableland and Victoria were greater than expected by chance (p=

<0.001). There was a statistically significant higher BMSCC in the Victorian herds compared with those tested on the Atherton Tableland.

Table 9.1 Association between somatic cell count and presence of *Mycoplasma bovis* in the bulk milk from 186 dairy herds on the Atherton Tableland, far north Queensland.

Mastitis level[1]	SCC x 10^3 /mL	No. of bulk milk samples at each level		No. of bulk milk samples positive by MB-PCR	
Slight	<250	72	(39%)	23	(32%)
Average	250-500	101	(54%)	50	(49.5%)
Above average	>500	13	(7%)	8	(61.5%)

[1] O'Rourke and Blowey 1992

Table 9.2 Association between somatic cell count and presence of *Mycoplasma bovis* in the bulk milk from 165 dairy herds from Victoria.

Group	SCC x 10^3/mL	No. of bulk milk samples at each level		No. of bulk milk samples positive by MB-PCR	
Slight	<250	46	(28.5%)	15	(33%)
Average	250-500	91	(56%)	65	(71%)
Above average	>500	28	(17%)	23	(82%)

There was a significant relationship between a high SCC (≥250,000 SCC/mL) and detection of *M. bovis* PCR in the bulk milk for both the Atherton Tableland and Victoria (Chi-square test, $p \leq 0.001$, $\chi^2 = 25.232$ with 2 degrees of freedom and $p \leq 0.006$, $\chi^2 = 10.181$ with 2 degrees of freedom for Atherton Tableland and Victoria respectively).

9.3.2 Composite milk

The association between detection of *M. bovis* and SCC in composite milk from Farm 1 is shown in Table 9.3 (the raw data is shown in Appendix 5). The most interesting observation was the 95% association of *M. bovis* with SCC >10^6. There was a 50-70% prevalence for the other SCC groups demonstrating the high prevalence of *M. bovis* on this farm.

Table 9.3 The association between somatic cell count and the presence of *Mycoplasma bovis* in individual cow composite milk samples from Farm 1.

Group	SCCx10³/mL	Milk samples (n=90)		Milk samples *M. bovis* PCR positive	
Slight	<250	45	(50%)	25	(55.5%)
Average	250-500	17	(18.8%)	12	(70.5%)
Above average	500-750	6	(6.6%)	4	(66.6%)
Bad	750-1000	2	(2.2%)	1	(50%)
Severe	>1000	20	(22.2%)	19	(95%)

9.3.2.1 Temporal association of M. bovis and SCC

The persistence of *M. bovis* infection was determined by testing monthly milk samples. In most cases the association of *M. bovis* with elevated monthly SCC was consistent. These results are shown in Tables 9.4 and 9.5. Again there was a clear association between *M. bovis* (PCR positive) and an elevated SCC on both farms. On Farm 1, a number of animals with a low or normal SCC were shown to be excreting the organisms. Also some animals were found to shed *Mycoplasma* intermittently. Two animals with a high SCC that were positive for *M. bovis* were found to be negative at later testing, and one animal with a high SCC that was negative for *M. bovis* was later found to be positive. On the other hand, two animals with low SCC that were positive for *M. bovis* were negative for *M. bovis* at a later test and two other animals with a low SCC converted from *M. bovis* negative to *M. bovis* positive. Moreover, in one case the SCC increased markedly in an animal which remained negative for *M. bovis* by PCR. However, in most cases the positive PCR results were consistent with an elevated SCC.

9.3.3 Bacteriological culture

The bacteria isolated from milk samples were categorised as MMP and non-MMP. Major mastitis pathogens included *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*. Non-MMPs included *Serratia marcessans, Staphylococcus* spp., *Corynebacterium bovis, Streptococcus uberis, Enterobacter cloacae, Neisseria morganii, Klebsiella oxytoca, Klebsiella pneumoniae, Micrococcus* spp., *Pseudomonas* spp., *Bacillus* spp., *Enterobacter agglomesans* and *Citrobacter freundii.* The raw data is tabulated in Appendix 6.

Table 9.4 Temporal association between *Mycoplasma bovis* and SCC in composite milk samples from individual cows from Farm 1.

	Date: 27.3.96		Date: 26.4.96	
Cow No.	SCC x 10^3/mL	PCR Result	SCC x 10^3/mL	PCR Result
82	7410	0*	7040	0
11	7065	0	168	0
64	6717	+	3400	–
257	6392	0	201	0
50	5818	0	787	0
64	5445	0	3400	0
61	3711	0	6649	0
244	3097	0	915	–
82	1081	–	615	+
10	456	0	1429	+
45	128	–	260	–
206	75	–	61	–
259	72	–	124	–
229	71	–	34	+
249	70	0	315	+
33	49	–	148	+
173	46	–	36	–
229	46	–	34	–
86	42	0	93	–
79	41	0	36	+
97	34	–	1077	–
58	14	0	11	–

* Not tested

Table 9.5 Temporal association between *Mycoplasma bovis* and SCC in composite milk samples from individual cows from Farm 3.

	SCC *	SCC	SCC	PCR Test	DNA Probe	SCC	PCR Test
Cow no.	15/9/95	10/11/95	11/1/96	7/2/96	7/2/96	12/2/96	20/3/96
47	1504	2511	226	+	+	301	+
88	1496	420	1105	+	+	2477	+
330	190	347	1759	+	+	476	+
420	219	372	231	+	+	351	+
473	776	604	743	+	+	1024	+
516	104	906	346	+	+	775	+
755		241	2500	+	+	532	+
1036	320	397	545	+	+	242	+

* SCC × 10^3/mL

9.3.4 The association between *M. bovis* and persistently high somatic cell count

Of the 202 quarters from cows with persistently high SCC, two quarters with clinical mastitis and producing obviously abnormal milk that was watery or serous, yellow to brown in colour and with flaky sediment were observed (Figure 9.1). A cow was considered positive for *M. bovis* if *M. bovis* was detected in one quarter. Thus 76.9% of animals were positive, of which 19.2% had *M. bovis* alone without any other bacteria, 17.3% had *M. bovis* in combination with MMP and 40.38% had *M. bovis* in combination with non-MMP bacteria.

At the quarter level *M. bovis* was detected in 36% quarter milk samples tested, of which 19.8% had *M. bovis* alone without any other bacteria, 5.4% had *M. bovis* in combination with MMP and, 10.8% had *M. bovis* in combination with non-MMP bacteria. No bacteria were detected in 42% of quarters. The summary data is shown in Table 9.6 and the raw data is tabulated in Appendix 6.

Figure 9.1 Four quarter milk samples demonstrating typical clinical *Mycoplasma* mastitis obtained from cows with clinical mastitis refractory to treatment on the Atherton Tableland. They were positive by MB- PCR. Sample 1: Abnormal milk with brown colour from one remaining functional quarter of cow, the other three quarters were blind. Samples 2, 3 and 4: Abnormal milk characterised as watery with sediment present from three functional quarters of one cow in which the fourth quarter was blind.

Table 9.6 The presence of *Mycoplasma bovis* determined by *Mycoplasma bovis*-polymerase chain reaction and other bacteria cultured from the quarter milk of cows with persistently high somatic cell counts.

Level of infection	No. of Samples	*M. bovis* alone	*M. bovis* with MMP	*M.bovis* with non-MMP	MMP only	Non-MMP only	No *Mycoplasma* or bacteria
Quarter	202	40(19.8%)	11 (5.4%)	22 (10.8%)	18 (8.9%)	26 (12.8%)	85 (42%)
Cow	52	10(19.2%)	9 (17.3%)	21 (40%)	3 (5.8%)	5 (9.6%)	4 (7.7%)

9.3.5 Herd visits

9.3.5.1 Clinical observations

On Farm 1, clinical mastitis was observed in two cows, pneumonia in one calf and keratoconjunctivitis in one cow. On Farm 2, no clinical signs of disease were observed. Nevertheless some cows had recorded a high SCC in a recent herd test and some of them were reported to have suffered from poor fertility.

9.3.5.2 Mycoplasma detected in milk by PCR and by culture

Mycoplasma bovis was detected in 92.8 and 66% of cows from Farm 1 and Farm 2 respectively (Table 9.7). Of 113 quarter milk samples collected at the farm visits, 92 were cultured for *Mycoplasma.* In three cases from Farm 1 abnormal milk that was watery with flaky sediment was observed (Figure 9.1) and found to be *M. bovis* positive. *Mycoplasma bovis* was isolated from two quarters of one cow with clinical mastitis on Farm 1 (Figure 9.2). The remaining samples were culture negative. When samples were tested by PCR, 35 (40%) of quarters were positive.

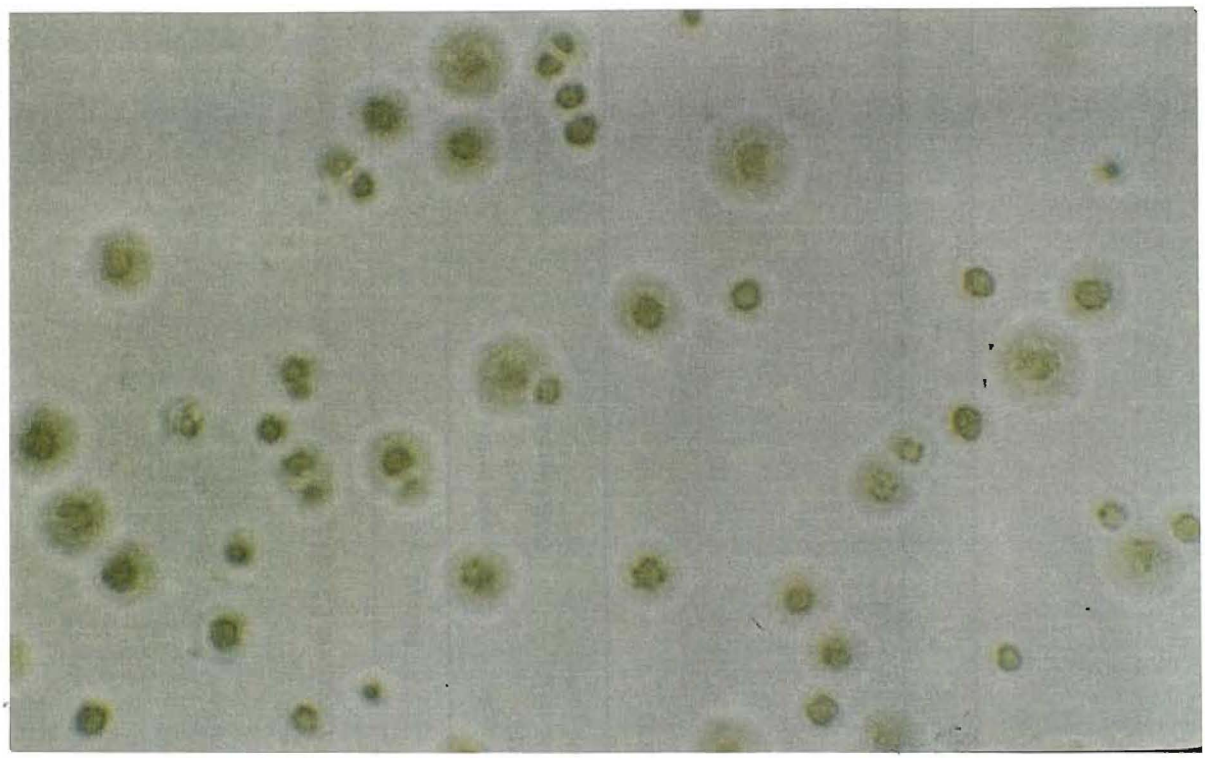

Figure 9.2 Typical "fried egg" colonies of *Mycoplasma* isolated from a cow with clinical *Mycoplasma* mastitis on Farm 1 on the Atherton Tableland.

9.3.5.3 Serology

On Farm 1, 92.8% and on Farm 2, 73.3% of cattle had high reactivity of *M. bovis* antibodies detected in the indirect ELISA (Table 9.7). All the sera positive in the indirect ELISA showed more than >50% inhibition in the blocking ELISA except one animal that was equivocal with 40% inhibition. One animal on Farm 1 that was positive for *Mycoplasma* by culture and PCR and another cow on Farm 2 that was positive only by PCR, had low antibody titres by indirect ELISA and low % inhibition in the blocking ELISA.

In the indirect ELISA a titre of 256 correlated with 50-60% inhibition in the blocking ELISA. Thus, 256 was chosen as the cut-off for a positive reactor in the indirect ELISA.

Table 9.7 Association between antibodies to *Mycoplasma bovis* in cattle sera as detected by indirect and blocking ELISA with detection of *Mycoplasma bovis* by PCR on Farms 1 and 2.

		Farm 1		Farm 2		
Endpoint in indirect ELISA	**Percentage inhibition in blocking ELISA**	**Serum samples (n=14) Reactor at each ELISA endpoint**	**Composite milk (n=14) PCR+/sero-reactor**	**Serum samples (n=15) Reactor at each ELISA end point**	**Composite milk samples (n=15) PCR+/seroitive**	**Nasal swabs (n=15) PCR+/sero-positive**
32	<40	0	0	1 (6.6%)	0/1	1/1
64	40-50	1 (7.14%)	1/1	3 (20%)	1/3	2/3
256	50-60	7 (50%)	6/7	9 (60%)	7/9	7/9
1024	>60	6 (42.8%)	6/6	2 (13.3%)	2/2	2/2
Total			13/14 (92.8%)		10/15 (66%)	12/15 (80%)

9.3.5.4 Nasal swabs

Mycoplasma bovis was detected in the nasal secretions from the three calves tested on Farm 1 and from 12 of 15 cows tested on Farm 2 (Table 9.7). All cows with milk PCR positive for *M. bovis* had a positive nasal swab. Of the five cows with milk negative by PCR, two had nasal swabs positive by PCR.

9.4 Discussion

The main objective of a mastitis control program is to reduce and keep the number of infected quarters within a herd to a minimum. However, mastitis remains the most influential factor affecting the quality and quantity of milk produced (Reneau 1986). This is often despite the use of a number of management control measures including, teat dipping, dry-cow therapy, proper use of a functionally adequate milking system, correct milking technique, culling of chronically infected cows, prompt treatment of clinical mastitis and environmental sanitation (Colditz and Watson 1985). However, in a recent study by Hoblet et al. (1991) in nine herds that effectively controlled mastitis caused by *Streptococcus agalactiae* and *Staphylococcus aureus,* mastitis still affected 16 to 64% of quarters. Thus it seems that the most important single factor preventing progress in mastitis control is the lack of effective therapy for all of the aetiological agents.

As a general principle, it is believed that once a quarter becomes infected it remains so until there is spontaneous recovery, or else the cow is culled (Bramley and Dodd 1984). *Mycoplasma bovis* has been shown to persist in the mammary gland of naturally and experimentally infected cows (Jasper et al. 1966; Gonzalez and Sears 1994). Thus effective control of mastitis where this organism is implicated poses very special problems.

Identification of the causative pathogen or pathogens involved is fundamental to the investigation of any mastitis program in particular, as knowledge of the type of pathogen will provide very useful indicators as to the predisposing factors involved. *Mycoplasma* mastitis has been reported with increasing frequency worldwide (reviewed by Kirk and Lauerman 1994). However, culture which is currently used for the detection of *Mycoplasma,* is extremely insensitive (Simecka et al. 1992).The MB-PCR assay was developed using *M. bovis*-specific primers and adapted to determine the prevalence of *M. bovis* in bulk milk

samples, composite milk samples from individual cows and milk samples from cows with persistently high SCC.

A SCC of 250 x 10^3 cells/mL has been suggested as the benchmark above which infected cows can be identified (Dohoo and Meek 1982). The association of *M. bovis* with high SCC in bulk milk from the Atherton Tableland was 50.8%, from Victoria 73%, and using composite milk from individual cows from one farm on the Atherton Tableland an association of 80% suggests that this agent may play an important role in the aetiology of subclinical and chronic mastitis. Subclinically infected quarters usually have cell counts greater than 250 x 10^3 cells/mL and when the cell count exceeds 10 x 10^6 cells/mL, clinical signs would be expected (Dohoo and Meek 1982). The reason for negative *M. bovis* cases showing high SCC may be related to concurrent illness or perhaps inhibitor substances in the milk sample prepared for PCR or a different pathogen involved.

In this chapter the close relationship between high SCC and *M. bovis* confirmed the high sensitivity of the MB-PCR. *Mycoplasma bovis* was persistent over a period of one month in most samples. However a few samples showed differences between *M. bovis* PCR result, perhaps because of intermittent shedding of *M. bovis*, as previously reported (Jasper 1981) or may be they were new infections or spantaneaus recoveris. However, this finding indicates that to detect individual animals infected with *M. bovis,* frequent testing may be required.

A similar immunosuppressive effect of *M. bovis* infection has been reported (Bennett and Jasper 1977a).There is evidence from the present study that *M. bovis* infection is widespread in dairy herds and this may increase the susceptibility of infected quarters to bacterial infection. Only *M. bovis* was detected in 19.8% of milk samples with persistently high SCC taken from six farms. In 57.7% of cases, *M. bovis* was detected with other bacteria. In only 15.4% of cases, were bacteria other than *M. bovis* isolated without detection of *M. bovis* and in 7.7% no bacteria were cultured and *M. bovis* was not detected.

As PCR detects the DNA of even non-viable organisms, the results are probably biased towards detecting *M. bovis*. The low recovery rate of bacteria on culture, commonly encountered during an acute inflammatory process, may also contribute to these results. Nevertheless, bacteria such as *Streptococcus agalactiae*, *Staphylococcus aureus* could be

considered secondary invaders acting synergistically with *M. bovis* in the development of mastitis.

In support of this proposal it has been reported that in herds chronically infected for several years, *M. bovis* infection can increase the mastitis infection rates caused by other pathogens and environmental microorganism (Bayoumi et al. 1988; Brown et al. 1990). In a report on a herd with a high BMSCC, 52 to 65% of cows were culture positive for *Streptococcus agalactiae* and *Staphylococcus aureus* but there was no response to intra-mammary therapy, and the clinical picture worsened. After several weeks most of the cows were culture positive for *M. bovis* (Gonzalez et al. 1992). In another study, *M. bovis*, as a cause of, or associated factor in, mastitis was reported in a large herd of 1400 lactating cows. Two groups of cows were identified based on their *M. bovis* infection status. The percentage culling rate due to low production and mastitis was higher in cows infected with *M. bovis* compared with uninfected cows (Brown et al. 1990). Further support comes from a study by Polner et al. (1989) who showed that cattle previously infected with *Mycoplasma* developed more severe clinical mastitis when they were inoculated with *Streptococcus dysgalactiae*. There is thus convincing evidence from the literature for the significant association of *M. bovis* with mastitis acting either directly or synergistically with recognised mastitis pathogens.

The synergism of *M. bovis* with other potential udder pathogens may be linked to the immunosuppression effect of *M. bovis* infection reported by Bennett and Jasper (1977a). Furthermore, *M. bovis* is able to inhibit the *in vitro* killing of bacteria (Howard and Taylor 1983) and reduce neutrophil activity (Thomas et al. 1991).Other mycoplasmal infection (*M. suipneumonia*, *M. gallisepticum* and *M. pneumoniae*) have also been reported as having an immunosuppressive effect (Ciprian et al. 1988; Ross and Young 1993; Cimolai et al. 1995).

The respiratory tract has been suggested as a route for transmission of *Mycoplasma* infection to the udder (Bennett and Jasper 1977b) and thus nasal secretions were tested. A high prevalence of *M. bovis* was found in the respiratory tract of cattle and calves tested. It was interesting that all cows that had milk positive for *M. bovis* also had positive nasal swabs. However of the five cows where milk was negative, only two had positive nasal swabs and also these two had low antibody titres to *M. bovis* in the two ELISA tests. It is tempting to speculate that the infection may be acquired by the respiratory route and then transmitted to

the udder, joints and reproductive tract by the haematogenous and/or lymphatic route. In several previous studies, *M. bovis* has been isolated from the nasal cavity of cows in herds where *M. bovis* mastitis has occurred (Bennett and Jasper 1977b). Calves infected with *M. bovis* in the upper respiratory tract may develop pneumonia when stressed or become infected with other pathogenic microorganisms (Stalheim and Proctor 1976; Ross 1985). Calves may become infected *in utero* or postpartum with *M. bovis* and it may inhabit the respiratory tract of these animals until the first calving, resulting in a closed infection cycle (Ter-Laak et al. 1992b). This hypothesis is strengthened by the observation that *M. bovis* has also been isolated from heifers that have never been milked (Jasper 1981) and from the sperm of young bulls (Pfutzner and Schimmel 1985). All these features would contribute to the ready transmission of *M. bovis* within a herd.

Despite the administration of broad spectrum antibiotics and implementation of milking sanitation, *M. bovis* infection persists and infected cows shed *M. bovis* in their milk intermittently, for months in some cases (Jasper et al. 1966; Jasper 1977; Gonzalez and Sears 1994). The SCC is reduced significantly in some cases but after an interval of months was elevated again. This suggests that *M. bovis* persists at the site of infection and antibiotic therapy removes the secondary bacteria only. This feature is one characteristic of *Mycoplasma* infection that probably relates to a defence system not being very effective against the infection (Adegboye 1978). This phenomenon has been reported for several other *Mycoplasma* infections. *Mycoplasma mycoides* subsp. *mycoides* has been isolated from CBPP lesions at least 10 months old (Turner 1954) and *M. hyopneumoniae* has been demonstrated in pig lungs as late as 262 days after primary infection (Whittlestone 1972). Similarly, *M. pulmonis* was regularly isolated from rat lung from 50 to 715 days after inoculation (Whittlestone et al. 1972). These observations suggest that a radical approach to mastitis control may be required and possibly the culling of infected animals will be the only effective means of control in a herd. Studies on *M. bovis*-free herds are required to investigate this proposal.

In this study and a report by Wentink et al. (1987), *M. bovis* was isolated from milk both with an increased or normal SCC. Detection of *M. bovis* in cows with normal SCC may be related to collection of the sample at an early stage of the infection before an inflammatory response occurs. A SCC is used to identify cows or herds with mastitis. It provides a fair estimate of the subclinical mastitis status of the herds. As reported previously (Jasper 1981) and also shown

in this study, *Mycoplasma* infection may or may not produce an inflammatory reaction. Thus, SCC will not accurately detect all udders infected with *Mycoplasma*. Furthermore, *M. bovis* was detected by MB-PCR in some cattle which were negative by blocking ELISA. This finding suggests that detecting *M. bovis* might be possible prior to a humoral immune response. However further testing is required to confirm this observation.

A high association was found between positive reactors detected by indirect or blocking ELISA and animals positive by MB-PCR. The high reactivity of *M. bovis* antibodies in the cattle sera was consistently high, which indicated active infection. Thirteen of 14 cows were positive both by PCR and serology on Farm 1, also 11 of 15 by serology and 10 of 15 by PCR from Farm 2. Nevertheless, *M. bovis* was isolated from only one of these 29 cows. Using cultural methods this cow also had a low antibody titre. This finding reconfirms the very low sensitivity of culture reported by Simecka et al. (1992), and that *M. bovis* infection is not always associated with an antibody response (Bennett and Jasper 1978a; Jasper 1981).

These studies have confirmed the high sensitivity and specificity of the MB-PCR developed in Chapter 8 and revealed a previously unrecognised prevalence of *M. bovis* in dairy herds in north Queensland and Victoria. These initial studies show a clear association between *M. bovis* and elevated SCCs. The likelihood that *M. bovis* is a major cause of, or predisposing factor for, bovine mastitis requires further elucidation.

CHAPTER 10

GENERAL DISCUSSION

10.1 Introduction

Mycoplasma are widely distributed in nature and numerous species are recognised (Maniloff et al. 1982; Razin and Barile 1985). They are among the most nutritionally fastidious microorganisms and can retain both extracellular and intracellular biological function (Taylor-Robinson 1991). They are reasonably host specific and a number of them have been reported to cause serious disease in their hosts. Others are associated with disease conditions but their potential to cause disease is unknown (Simecka et al. 1992). At least 12 species have been identified in cattle.

Frequently *M. bovis* has been isolated from healthy cattle, and from infected cattle with different clinical conditions (Jasper 1982; Bushnell 1984; Brown et al. 1990). Most of the infections caused by *M. bovis* are thought to be subclinical or chronic (Jasper 1982), however episodes of severe clinical cases and insidious spread of disease have also been reported (jasper 1981; Gunning and Shepherd 1996). It is believed that the subclinical and chronic carrier states are the more dangerous form in regard to the prevention and control of *Mycoplasma* disease because they allow the insidious spread of disease (Gourlay 1973). The pathogenicity of *M. bovis* infection has been reported for a variety of anatomical sites tested under controlled experimental conditions in gnotobiotic calves (LaFaunce and McEntee 1982; Bocklisch et al. 1986; Thomas et al. 1986; Jasper et al. 1987; Simecka et al. 1992). Of particular importance is that the reaction in the udder is severe and more persistent than the other sites. It is believed that the mammary gland is more sensitive to *Mycoplasma* infection than other tissues (Gourley and Howard 1979; Bushnell 1984).

A number of factors affect *Mycoplasma* infection in cattle. Some of significance include the route of infection, the age and immune status of the cattle, virulence of the *Mycoplasma* strain and antigenic variation (Simecka et al. 1992; Behrens et al. 1994; Rosengarten et al. 1994).

Very little work has been reported on *M. bovis* in dairy cattle since Cottew (1970) first isolated the organism from milk in Australia. Rapid, specific and sensitive laboratory diagnostic techniques are necessary to effectively detect *Mycoplasma* infection (Simecka et al.

1992). The present study was conducted to develop improved diagnostic techniques for the detection of *M. bovis* and its discrimination from other *Mycoplasma* spp. and other bacterial species causing infections in cattle.

10.2 Isolation of *Mycoplasma bovis*

The routine diagnosis of *M. bovis* infection is currently by conventional culture methods followed by serological identification of the isolate. Although culture of *Mycoplasma* spp. has been suggested for the diagnosis of *Mycoplasma* infection (Jasper 1981), this method is extremely insensitive and carrier animals may go undetected and clinical infections undiagnosed. It has been suggested that microbiological culture is not a suitable screening method to detect *Mycoplasma* infection in the respiratory or genital trat because of difficulties in obtaining suitable tissue or mucus samples (Thomas et al. 1987b). This may be related to the invasiveness of *M. bovis* (Howard et al. 1987), with penetration deep into the basal membrane making access to them impossible. In this study, despite the macroscopic and microscopic appearance of lesions suggesting *Mycoplasma* infection in the lungs sample, *M. bovis* was isolated from only 7.5% of lungs with pneumonia (Chapter 4) although this would not be attributable to invasiveness. Of the 15 cases examined by histopathology and found to have lesions resembling *Mycoplasma* pneumonia, *M. bovis* was isolated from only three. This gave an isolation rate of 20% suggesting low sensitivity of culture isolation. In addition, culture was labourious and costly and bacterial contamination was common.. As *Mycoplasma* may be shed in low numbers, a week of incubation on agar plates or enhanced growth in broth and then passage on agar may be required before typical colonies can be identified. Another disadvantage of culture diagnosis is that the results are available only after several days, and may not be available for up to ten days in unfavourable circumstances. As a result, removal of cows with *Mycoplasma* infection can be delayed by the long interval required for a positive diagnosis. Furthermore, cows may intermittently shed *Mycoplasmas*. Repeated culture of milk from 35 cows that were positive at least once demonstrated that the organism is not readily isolated (Jasper et al. 1966). Despite the poor sensitivity and slow growth of the organisms, culture remains the only option in most laboratories for routine diagnosis of *M. bovis* infection in cattle.

In this study, culture of freshly collected quarter milk samples from clinically infected cattle yielded *M. bovis*, confirming the need for high quality samples for culture. Despite the

inherent difficulties with the methodologies, the possibility of culturing *Mycoplasma* from infected animals is not only of great help in diagnosis, but also it provides confirmation of the presence of the pathogen and may enable a better understanding of the disease.

As a conventional alternative to antigen detection, antibodies against *M. bovis* in blood and milk can be detected by ELISA. This technology is less labour-intensive than culture and allows the screening of large numbers of samples. Results are normally available within 24 hours.

10.3 Development of Serological Assays

The commonly used serological assays for *Mycoplasma*, are complement fixation, IHA, HI and metabolic inhibition. However, these methodologies are relatively insensitive for the detection of the primary immune response against infectious mycoplasmas (Simecka et al. 1992; Regalla 1995). Immunoblot procedures are complex and costly, with requirements for the culture of the *Mycoplasma* for preparation of the antigen. The indirect ELISA for detection of antibody to *Mycoplasma* infection is suggested to be an inexpensive, sensitive and rapid alternative for screening large numbers of animals (Davidson et al. 1981; Busolo et al. 1983). The indirect ELISA was developed to screen for *M. bovis* antibody in animal and human sera used in and during the production of MAbs to *M. bovis*. The limitation of the system includes the extensive purification of antigen required and non-specific background signals.

In the present study, a lack of antigen stability and cross-reactions originating from antigens shared by other *Mycoplasma* spp. were observed in the indirect ELISA. The indirect ELISA failed to discriminate unpurified antigen. While lacking specificity, under optimal conditions, the indirect ELISA is fast, reproducible, convenient and a relatively sensitive method for the detection of *M. bovis* antibody in animal sera. The high level of antibodies to *M. bovis* detected in this study in cattle sera, compared with other animals, indicates that cattle may be commonly infected with *M. bovis*.

10.4 Production of Monoclonal Antibodies

The indirect ELISA provided an excellent screening test for the production of MAbs. A panel of 15 MAbs was produced using whole-cell *M. bovis* antigen. Only one MAb (MAb E4) was specific to *M. bovis* while others were found to cross react with *M. arginini, M. agalactiae, M. bovirhinis, M.* group 7, *M. bovigenitalium* and *M. dispar*. The indirect ELISA using MAb E4 was highly specific for confirming the identity of *M. bovis* for the three *Mycoplasma* isolated from the lungs (Chapter 4) and one isolate from a quarter milk sample (Chapter 9). As documented by Simecka et al. (1992), this method was difficult to standardise for the direct detection of *Mycoplasma* antigen in clinical samples, being of low sensitivity and specificity.

Use of MAb E4 in an indirect ELISA configuration is a major development for the rapid identification of *M. bovis* from clinical isolates. Further work on more simple diagnostic tests including co-agglutination or latex particle reagents should be considered. A fluorescein isothiocyanate-conjugated reagent could also be developed to detect *M. bovis* in nasal secretions and possibly milk samples.

10.5 Development of a Monoclonal Antibody Blocking ELISA

The production of the novel MAb E4 provided the opportunity to develop a blocking ELISA to detect antibody to *M. bovis* in cattle sera. Monoclonal antibody E4 not only contributed to the specificity of the assay but also eliminated the background reactions. Better sensitivity and specificity of blocking ELISAs have been reported previously by a number of workers for the detection of *M. hyopneumoniae* (Feld et al. 1992), *M. gallisepticum* (Czifra et al. 1993, 1995) and *M.* F38 (Thiaucourt et al. 1994). The *M. bovis* antigen coated on polystyrene plates was blocked with PRS and cattle sera, reflecting the saturation of the available *M. bovis*-specific epitopes. The inhibition of binding of MAb E4 resulted from the presence of *M. bovis* specific antibodies in the cattle sera. The assay could be very useful for sero-epidemiological studies where species specificity is of prime importance.

To evaluate and validate the assay, the MAb blocking ELISA was used to test 100 sera from dairy cattle from the Atherton Tableland. Sixty percent of animals were found to be positive revealing a high seroprevalence of *M. bovis* in the herd tested, a possible reflection of the

status of herds in north Queensland. This high infection rate in dairy herds is corroborated by the high level of *M. bovis* detected by PCR (Chapters 8 and 9).

However, not all *M. bovis* infected animals will be detected by blocking ELISA as antibody titres only emerge ten to fourteen days after the onset of disease. Furthermore, *M. bovis* infection of cattle is apparently not always associated with the production of an antibody response (Bennett and Jasper et al. 1978a; Jasper 1981).

The poor sensitivity of culture for diagnosing *M. bovis* infection may be related to high levels of circulating or secretory antibody specific to *M. bovis* (Chapter 3 and Chapter 9). The use in media of native sera with high titres of antibody to *Mycoplasma* was shown in Chapter 3 to inhibit the growth of *M. bovis* and *M. arginini*. After acid treatment of the serum to remove these antibodies, the *Mycoplasma* were successfully grown. Moreover, *M. bovis* was isolated from one cow with clinical mastitis that showed a low level of circulatory antibody to *M. bovis* (Chapter 9) which may have reflected a low level of antibody in the milk, thus facilitating the isolation of the organism. In a small pilot study, 24 milk samples were tested by blocking ELISA for antibody to *M. bovis*. Twelve samples gave greater than 50% inhibition. While this suggested that there was indeed antibody in the milk, no further work was undertaken in this project but it should be the focus of further study.

From this study it is concluded that samples for *Mycoplasma* culture should be obtained at an early stage of infection and that it is very difficult to isolate *Mycoplasma* after the appearance of antibody. This conclusion is supported by a study by Jelinek et al. (1993) who failed to isolate *Mycoplasma* from bulk-milk, concluding that *Mycoplasma* mastitis is rare in dairy cattle in Western Australia. Antibody present in the bulk milk may have precluded the isolation of *M. bovis* in this study. In addition sterilised bulk milk was inoculated with viable *M. bovis* as their positive control. As antibody would be destroyed by heat, the use of sterilised milk is considered an inappropriate control for use in this study, and probably led to an incorrect conclusion. Further support for the suggestion that antibody in milk precludes isolation of the organisms is provided by reports that the high concentration of specific antibodies in the synovia prevent the isolation of *Mycoplasma* (Frey 1986). However, *Mycoplasma* should be detected by PCR as the DNA-based detection method would be unaffected by the presence of antibody.

10.6 Development of a DNA Probe

A DNA probe for the detection of *Mycoplasma* should have several advantages over serological dn culture methods. These advantages would include high specificity and sensitivity and the ability to detect targets that may demonstrate antigenic variation. Moreover, a DNA probe would be stable and could detect *Mycoplasma* infection before the appearance of a specific serological response (Falkow 1985). DNA probes for detection of *M. bovis* have been reported (Mattsson et al. 1991; McCully and Brock 1992) but they lacked specificity, mainly because of cross reactivity with *M. agalactiae* and *M. arginini* respectively. The specificity and sensitivity of a DNA probe is also dependent upon such parameters as temperature, salt concentration, hybridisation wash temperature, pH of the hybridisation reaction buffer, the property of the probes and exposure time of the radiolabelled probe to the film (Sambrook et al. 1989). All these parameters were optimised and the novel probe *Mb* 1-6 developed in this study reacted strongly with the genomic DNA of the *M. bovis* reference strains and moderately with the *M. bovis* Oonoonba strain, but not with any other *Mycoplasma* or bacterial DNA tested. The sensitivity of the dot blot hybridisation assay was 200 CFU/mL.

10.7 Development of MB-PCR

To enhance sensitivity further, *M. bovis*-specific PCR assay was developed. The first step in evaluating the usefulness of a PCR for *Mycoplasma* detection was to establish the PCR protocol. This involved the design of *M. bovis* specific primers for amplification and the optimisation of the parameters of the reaction to provide the greatest sensitivity and specificity. A protocol requiring minimum sample manipulation was considered important to minimise time.

Specificity and sensitivity of any PCR assay lies almost entirely with the design of the oligonucleotide primers. However due to the limited published sequence data for *M. bovis*, the primers were designed using sequences obtained from the specific DNA probe (Chapter 7) which discriminated *M. bovis* from all other *Mycoplasma* DNA. The primers were selected from flanking regions of this probe based on high and compatible primer annealing temperatures and low self- and inter-primer complementarity. No significant homology with any other microorganism was found, suggesting that these primers were unlikely to cross-react with other bacterial DNA.

Having selected the primers, optimisation of annealing temperature, cycle number and $MgCl_2$ concentration was performed to maximise sensitivity and specificity. Various methods of improving sensitivity were also assessed. Once fully optimised the assay was used to detect *M. bovis* in clinical samples.

10.8 Application of MB-PCR

Detection of *Mycoplasma* in clinical samples should give an unequivocal diagnosis of *Mycoplasma* infection. Polymerase chain reaction is now used as an aid in the diagnosis of many infections including *M. hyopneumoniae* (Mattsson et al. 1995), *M. agalactiae* (Tola et al. 1996), *M. pneumoniae* (Luneberg et al. 1993), *M. meleagridis* (Zhao and Yamamoto 1993), *M. mycoides* subsp. *mycoides* SC (Bashiruddin et al. 1994) and *M. bovis* (Hotzel et al. 1993, 1996). The MB-PCR developed in this study could be used for the first time as an aid in preliminary studies of *M. bovis* infection in dairy cattle due to its high sensitivity (20-30 CFU/mL *M. bovis*) and ability to discriminate *M. bovis* from other *Mycoplasma* species (Chapter 8). It was more specific, sensitive and rapid than the PCR developed by other workers (Hotzel et al. 1993, 1996) for detection of *M. bovis* in clinical samples. The MB-PCR was used in this study to detect *M. bovis* in milk samples and correlate the presence of *M. bovis* and high SCC, the cause of down-grading of milk quality. Furthermore, it was used to detect *M. bovis* on nasal swabs as an investigation of the respiratory route in the spread of infection.

10.9 Preliminary Studies on the Prevalence of *Mycoplasma bovis* Mastitis in Dairy Cattle

The observation that *Mycoplasma* DNA could be detected by PCR (Chapter 8) from bulk milk provided a valuable method for determining the presence of this organism in a herd without requiring the culture of milk samples from large numbers of cows. A negative bulk milk was not regarded as proof that infection was absent in the herd but was considered to imply a low level of infection or minimal-severity as compared with herds where bulk milk readily yielded a PCR positive result. *Mycoplasma* DNA could be detected with high frequency from bulk milk samples, particularly in herds with higher SCC. This PCR is therefore useful for monitoring the *M. bovis* status of a herd on a temporal basis.

Somatic cell counts are used to identify cows or herds with mastitis. Milk from a healthy, uninfected bovine mammary gland should be sterile (Bramley 1992) and contains somatic cells comprising macrophages, neutrophils, lymphocytes and epithelial cells, usually $<250 \times 10^3$ cell/mL milk. When mastitis is present, the cell count increases, primarily because of neutrophil infiltration. Subclinically infected quarters usually have cell counts greater than 250×10^3 cells/mL, but because of the dynamic nature of the disease, this value can vary considerably and will sometimes fall below this threshold. During clinical episodes, the cell count will be elevated with counts greater than 10×10^6 cells/mL being common (Dohoo and Meek 1982). A high level of subclinical infection in a herd will result in a consistently high bulk milk cell count. In herds with counts of 250×10^3 cells/mL in bulk milk, there is generally little economic impact associated with subclinical mastitis.

The studies undertaken on bulk milk showed a significant correlation between high SCC and *M. bovis* infection. These preliminary findings suggest that *M. bovis* may cause, or perhaps predispose the udder to subclinical or clinical mastitis.

Individual cow cell counts were carried out to identify chronically infected cattle and to investigate its association with *M. bovis* infection further.

There was again a significant association of *M. bovis* with increased SCC. Thus, it is believed that *M. bovis* has a principal role in infection of the udder. *Mycoplasma bovis* has been reported to be associated with clinical, subclinical and latent forms of mastitis with high, moderate and normal SCC respectively (Kirk and Lauerman 1994). In this study (Chapter 9) the most prevalent form was subclinical mastitis. Subclinically infected animals are the main source of *Mycoplasma* infection in a herd as they continually shed *Mycoplasma* in body secretions and excretions. Jasper (1982) reported that poor management would enhance the spread of infection or would increase the severity of disease. Nevertheless, despite improved management, dairy cattle may still suffer from *M. bovis* infection. This is consistent with the observation of a higher level of *M. bovis* in Farm 1 compared with Farm 2, which had poor and high standards of management respectively.

Mycoplasma bovis was detected in milk from clinically affected quarters (Chapter 9). These animals all had consistently high composite milk SCC and MMP were rarely isolated. *Mycoplasma bovis* was detected by PCR in nearly 77% of the animals with MMP in only 17%

of animals, but many had N-MMP. These findings further support the view that *M. bovis* has the potential to produce disease alone or predispose the udder to disease caused by both major mastitis and environmental pathogens. Most of the infected cows did not have the obvious clinical signs of *Mycoplasma* infection described in the literature and were unnoticed in the herd except for their high SCC. Only in some cows were the clinical signs of *Mycoplasma* mastitis obvious, but these animals were *Mycoplasma* culture-negative. This again confirms the poor sensitivity of culture.

The MB-PCR could also detect *M. bovis* in the nasal cavity of cows and there was a close association between raised antibody titre and *M. bovis* in the milk. The high correlation of *M. bovis* infection of the respiratory tract and the udder of cattle suggests a probable route of infection. Thus, a mastitis control program involving udder teat dipping and individual therapy would be inappropriate for the control of *M. bovis* infection.

The high prevalence of *Mycoplasma* as diagnosed by PCR, may be related to detection of immune complexes that may produce auto-immune manifestations or be responsible for immunopathological lesions in the host. This phenomenon may be related to the high affinity of *Mycoplasma* for the plasma membrane of mammalian cells (Bredt 1976) and/or antigenic exchange with host cells (Bradbury and Jordan 1971; Sethi and Brandis 1972) which may cause an immune response against self antigens.

Finally the conclusions to be drawn from these studies are that the PCR was superior to all other conventional assays available because of its high sensitivity, specificity, convenience in terms of labour, cost and time, and its ease and range of application. The high antibody titre to *M. bovis* detected by ELISA correlated with a high frequency of detection of *M. bovis* by MB-PCR from animals with high SCC and clinical mastitis and indicates that *M. bovis* has an important role in subclinical disease in cattle and may predispose cattle to other pathogens. *Mycoplasma bovis* was widespread in the dairy herds tested both in north Queensland and in the areas of Victoria sampled. From time to time the organism may produce clinical disease alone or in conjunction with other bacteria. Detection of *M. bovis* in lung lesions exhibiting interstitial pneumonia and on nasal swabs from cows with high SCC and high antibody titres support the hypothesis that the respiratory tract is the reservoir of *M. bovis* infection, and that infection can spread through the body haematogenously and produce subclinical pneumonia, mastitis, arthritis and genital disease.

10.10 Future Research

The MB-PCR was very useful for detecting *M. bovis* infection in clinical samples. It could also be used for large scale epidemiological surveys. Characterisation of *M. bovis* in dairy herds and detailed epidemiological studies could provide useful information about its role in disease of cattle and the better control of these diseases.

Prevention of *M. bovis* infection poses challenging technical problems because basic information regarding disease pathogenesis is incomplete in many areas including the basis immune mechanisms that protect cattle against infections of the respiratory, mammary gland, urogenital and synovial systems, the pathogenesis of *M. bovis* infection, and also the disease related antigens and the protective immunogens to induce protection against *M. bovis* infection.

In particular, the role of *M. bovis* in the pathogenesis of bovine mastitis requires detailed study. This would lead to improved treatment of clinical mastitis and control of the disease.

Regulation of the host immune response by *M. bovis* is complex and studies are required to determine how *M. bovis* infection can persist despite the presence of intense immune and inflammatory responses. Developing a *M. bovis* vaccine would be desirable because of the high prevalence of *M. bovis* infection in dairy cattle, the protracted course of *M. bovis* infection, and the failure of antibiotics and other therapeutic approaches to eradicate the *M. bovis*.

The immune response to *M. bovis* infection may play a critical role in the pathogenesis of the disease and may be responsible for the histopathological lesions and the auto-immune manifestations seen in other *Mycoplasma* disease. Thus, developing engineered immunogens capable of raising a protective response without the components that induce the intense cell recruitment that participates in the disease process may be necessary.

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APPENDIX 1

Mycoplasma Media and ELISA Reagents

1.1 Mycoplasma wash solution (MWS)

0.25 M NaCl

20 mM Na_2PO_4

10 mM $MgSO_4$

1.2 Glycine buffer

0.27 M Glycine

Adjust to pH 1.5

1.3 Rabbit broth

Collect 125 grams of heart and skeletal muscle. Homogenise in one litre of dH_2O. Boil for 20 min then centrifuge at 3000 *g*. To the 1 litre of supernatant add:

Bacto peptone	10 g
NaCl	5 g

To 300 mL of this solution add 200 mL Hanks solution [Basal Medium Eagle with Hanks Salts (CSL, Melbourne)]
Autoclave at 121 °C for 15 min.

After autoclaving to 50 mL of solution add:

Yeast Extract (Chapter 4)	1 mL
Glucose (10% w/v)	1 mL
DNA (sodium salt from salmon testes) (Sigma Chemical Co) (0.2% w/v)	0.5 mL
Rabbit serum	10 mL

Adjust the pH to 7.6. Filter sterilise and store at -20°C.

1.4 10 x PBS (phosphate buffered saline) (per litre)

NaCl	30 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g
Distilled water	1000 mL

Stir at 4 °C until dissolved.

1.5 Tris buffer

Tris-HCl	0.6055 g
Distilled water	1000 mL

Adjust pH to 7.4 or 8.0 as required.

1.6 Regeneration buffer

Solution 1:

Acetic acid	6.005 g
Distilled water	1000 mL

Solution 2:

NaCl	81.82 g
Isopropanol	400 mL

Combine 600 mL of Solution 1 with 400 mL of Solution 2.

1.7 ELISA coating buffer

Na_2CO_3	1.378 g
$NaHCO_3$	3.108 g
Distilled water	1000 mL

Ajust pH to 9.6 and autoclave at 121 °C for 20 minutes.

1.8 10 x PBS Tween 20

To 1 litre of 10 x PBS add 4.4 mL of Tween 20 and stir to dissolve.

1.9 TEN-Tween casein (TEN-TC) x 10

Tris-HCl	60.55 g
EDTA	3.7 g
NaCl	87.7 g
Tween 20	5 mL
Distilled water	1000 mL

Stir to dissolve. Adjust the pH to 8.0, then add:

Casein	20 g

Stir in cold room overnight.

1.10 ELISA substrate solutions

ELISA substrate solution was prepared using 0.1 M citrate/phosphate buffer. For each ELISA plate 10 mL of citrate-phosphate buffer, 200 µL of ABTS stock solution and 200 µL H_2O_2 solution was used.

1.10.1 Citrate-phosphate buffer

Solution A (0.1M Citric acid)

Citric acid	15.75 g
Distilled water	750 mL

Solution B (0.2M Sodium phosphate)

Sodium phosphate	14.2 g
Distilled water	500 mL

Adjust pH of Solution B to 4.2 using Solution A.

1.10.2 H_2O_2 stock

Hydrogen peroxide (30% w/v)	63 µL
Distilled water	5 mL

Make fresh daily.

1.10.3 ABTS stock

ABTS	0.56 g
Distilled water	20 mL

Store in the dark.

1.11 Dienes stain

Methylene blue	2.4 g
Maltose	10.0 g
Azure II	1.25 g
Sodium chloride	0.25 g
Distilled water	100 mL

Stir until dissolved (Quinn et al. 1994).

APPENDIX 2

Monoclonal Antibody Production Reagents

2.1 Trypan blue stock solution (1.4%) for viable cell count

Trypan blue	1.40 g
Phosphate buffered saline	100 mL

Working solution is made by further diluting 1/10 in PBS

2.2 CSL medium

DMEM medium-High Glucose (Flow Laboratories Inc., USA; Cat. No. 1033124)	4.50 g
Non- essential amino acids for Eagle MEM (Iscove's MDM, Gibco USA. Cat. No. 430-2200EF)	1% of 100x
Sodium pyruvate	1 % of 100x
2-mercaptoethanol	5×10^{-5} M final conc.
Sodium bicarbonate	2.40 g
PSPK solution (Appendix 2.2.1)	10 mL
Fungizone solution (Appendix 2.2.2)	4 mL
Distilled water	to 1000 mL

Sterilise by filtration using 0.2 µm filter.

2.2.1 Antibiotic solution (PSPK)

Benzyl penicillin-Na salt	5×10^6 IU
Streptomycin sulphate	5.00 g
Polymyxin B sulphate	1×10^6 IU
Kanamycin Sulphate	2.00 g
Distilled water	250 mL

Dissolve and sterilise by filtration using 0.2 µm filter. Store at -20°C in 10 mL aliquots.

Fungizone solution

Fungizone [Amphotericin B, (Squibb)]	100 mg
Sterile distilled water	100 mL

Aseptically dissolve and store at -20°C in 4 mL aliquots.

2.3 New born calf serum (NBS)

Purchased from CSL, Melbourne, Australia Cat. No. 09252301.

2.4 Hypoxanthine aminopterin thymidine (HAT) x 100

Thymidine	0.038 g
Hypoxanthine	0.136 g
Aminopterin	0.0018 g
PBS	100 mL

Hypoxanthine is dissolved in PBS by adding a few drops of 1 M NaOH. Make the resulting solution up to 100 mL with PBS and add the other ingredients. Filter sterilise using a 0.2 μm filter, store at -20°C in 10 mL aliquots.

2.5 HT x 100

As for HAT x 100, omitting the aminopterin.

2.6 Acrylamide/bis (30% stock)

Acrylamide/bis	30 g
Distilled water	100 mL

Dissolve acrylamide/bis in distilled water. Filter sterilise using a 0.2 μm filter and store in dark bottle at 4°C. (Shelf life 30 days maximum).

2.7 1.5 M Tris-HCl (pH 8.8)

Tris base	27.23 g
Distilled water	80 mL

Adjust pH to 8.8 with 1 M HCl, then adjust volume to 150 ml with distilled water. Store at 4°C.

2.8 0.5 M Tris-HCl pH 6.8

Tris base	6 g
Distilled water	80 mL

Adjust pH to 6.8 with 1 M HCl, then adjust volume to 100 mL with distilled water. Store at 4°C.

2.9 SDS-reducing buffer (Sample buffer)

Distilled water	4.0 mL
0.5 M Tris-HCl, pH 6.8	1.0 mL
Glycerol	0.8 mL
10% (w/v) SDS	1.6 mL
2-β-mercaptoethanol	0.4 mL
0.05% (w/v) bromophenol blue in water	0.2 mL

Dilute the sample 1:3 with sample buffer, and boil for 5 minutes. Store sample buffer at room temperature.

2.10 Stock 5 x Electrode (Running) buffer (pH 8.3)

Tris base	9 g
Glycine	43.2 g
SDS	3 g
Distilled water	to 600 mL

Store at 4°C. Warm to 37°C before use if precipitation is present. Dilute 60 mL 5 x stock with 240 mL distilled water for use.

2.11 Coomassie Blue stain

Coomassie Blue	10 g
Methanol	400 mL
Acetic acid	100 mL
Distilled water	500 mL

2.12 Destain solution

Ethanol	200 mL
Acetic acid	200 mL
Distilled water	1600 mL

2.13 Western blot transfer buffer

Tris-HCl	3.03 g
Glycine	14.4 g
Methanol	200 mL
Distilled water	to 1000 mL

APPENDIX 3

Bacterial Media and DNA Extraction Reagents for Molecular Study

3.1 NTE buffer pH 7.4

5 M NaCl	20 mL
1 M Tris-HCl (pH 7.4)	20 mL
0.5 M EDTA (pH 7.5)	2 mL
Distilled water	to 1000 mL

3.2 TE Buffer

1 M Tris-HCl (pH 8.0)	1 mL
1 M EDTA (pH 8.0)	0.1 mL
Distilled water	98.9 mL

3.3 Loading buffer

Bromophenol blue (0.25%)	0.25 g
Xylene cyanol FF (0.25%)	0.25 g
Sucrose (40% w/v)	40 g
Distilled water	100 mL

Store at 4 °C

3.4 2 x YT medium

Bacto-tryptone	16 g
Bacto-yeast extract	10 g
NaCl	5 g
Distilled water	900 mL

Shake until dissolved. Adjust pH to 7.0 using 5 M NaOH. Adjust the volume of the solution to 1000 mL with distilled water. Autoclave 20 minutes at 121 °C.

3.5 2 x YT agar

2 x YT medium	1000 mL
Agar	20 g

Dissolve and autoclave for 20 minutes at 121 °C.

3.6 2 x YT ampicillin broth or agar

1 litre of autoclaved 2 x YT broth or agar

Cool to 55°C. Add ampicillin stock solution (Appendix 3.6.1).

3.6.1 Ampicillin Stock solution

Dissolve 150 mg ampicillin in 1 mL of distilled water. Sterilise by filtration through a 0.22 μ filter.

3.7 2 x YT agar containing ampicillin, X-gal and IPTG

To 500 mL of autoclaved 2 x YT agar cool gel to 55°C add ampicillin stock solution, X-gal solution and IPTG stock solution. Pour into Petri dishes.

3.7.1 X-gal stock solution

Make a stock solution (5-Bromo-4-chloro-3-indolyl-b-D-galactoside) by dissolving X-gal in dimethylformamide to make a 20 mg/mL solution. Place 2 mL aliquots in aluminium foil wrapped cryotubes and store at -20°C.

3.7.2 IPTG stock solution

Dissolve 2 g of IPTG (Isopropylthio-β-D-galactoside) in 8 mL distilled water. Adjust the volume of the solution to 10 mL with distilled water and sterilise by filtration through a 0.22 μ disposable filter. Aliquot 1 mL amounts and store at -20°C.

3.8 STE buffer

NaCl (0.1 M)	0.5844 g
1 M Tris-HCl (pH 8.0)	1 mL
1 M EDTA (pH 8.0)	0.1 mL
Distilled water	100 mL

3.9 GTE buffer

Glucose (50 mM)	9.0 g
1 M Tris-HCl (pH 8.0)	25 mL
1 M EDTA (pH 8.0)	10 mL

Solution I prepared in 100 mL amounts and autoclaved for 15 minutes at 121°C and stored at 4°C.

3.10 Lysozyme solution

Lysozyme	1 g
Distilled water	10 mL

Aliquoted in 1 mL microfuge tube and stored at 4°C.

3.11 Lysis buffer

NaOH (freshly diluted from a 10 M stock)	200 µL
SDS	0.1 g
Distilled water	10 mL

3.12 Potassium acetate solution

5 M potassium acetate	30 mL
Glacial acetic acid	5.75 mL
Distilled water	14.24 mL

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

3.13 Denaturing solution

NaCl (1.5 M)	87.66 g
NaOH	20 g
Distilled water	1000 mL
Distilled water	

3.14 Neutralising solution

NaCl	87.66 g
1 M Tris-HCl (pH 7.2)	500 mL
EDTA (0.001 M)	0.3722 g
Distilled water	500 mL

3.15 20 x SSC

3 M NaCl	175.32 g
$Na_3C_6H_5O_7.2H_2O$	88.23 g
Distilled water	1000 mL

3.16 50 x TAE (Tris-acetate) Concentrated stock solution

Tris-HCl	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA pH 8.0	100 mL
Distilled water	to 1000 mL

Autoclave at 121 °C for 20 minutes.

3.17 10 x PCR buffer

KCl	3.72 g
1 M Tris-HCl (pH 8.3)	1 mL
$MgCl_2$	0.40 g
Gelatin (0.01% w/v)	0.1 g
Distilled water	100 mL

Autoclave at 121 °C for 20 minutes and store at -20 °C.

APPENDIX 4

Table 4.1 Somatic cell count and MB-PCR Results of 186 Bulk Milk samples from the Atherton Tableland

Sample No.	SCC x 10^3/mL	PCR Results	Sample No.	SCC x 10^3/mL	PCR Results	Sample No.	SCC x 10^3/mL	PCR Results
1	691	–	45	376	–	89	293	–
2	625	–	46	373	+	90	293	+
3	606	+	47	370	–	91	290	–
4	582	+	48	367	–	92	288	–
5	582	+	49	365	+	93	283	–
6	579	–	50	365	+	94	282	–
7	559	–	51	361	–	95	280	–
8	533	–	52	360	–	96	279	–
9	518	+	53	359	+	97	278	+
10	517	+	54	356	–	98	271	+
11	516	+	55	356	+	99	270	–
12	510	+	56	354	–	100	268	–
13	509	+	57	353	+	101	267	+
14	498	–	58	350	+	102	267	–
15	492	+	59	349	–	103	265	–
16	481	+	60	348	–	104	263	+
17	481	+	61	344	–	105	263	–
18	480	+	62	342	+	106	259	–
19	467	–	63	338	–	107	259	+
20	460	+	64	330	–	108	257	–
21	459	–	65	327	+	109	255	–
22	458	+	66	327	+	110	254	–
23	454	+	67	325	–	111	254	–

Table 4.1 (cont'd)

Sample No.	SCC x 10^3/mL	PCR Results	Sample No.	SCC x 10^3/mL	PCR Results	Sample No.	SCC x 10^3/mL	PCR Results
24	451	–	68	324	+	112	254	+
25	447	+	69	324	+	113	253	–
26	442	–	70	324	+	114	252	–
27	439	+	71	323	+	115	248	+
28	437	–	72	322	+	116	245	–
29	435	–	73	320	–	117	244	–
30	433	+	74	320	–	118	243	+
31	430	+	75	319	+	119	243	–
32	427	+	76	319	+	120	240	–
33	423	+	77	316	–	121	236	–
34	422	+	78	314	–	122	236	+
35	420	+	79	312	+	123	234	–
36	410	+	80	311	+	124	234	–
37	409	–	81	308	–	125	229	+
38	407	–	82	307	–	126	226	–
39	406	+	83	306	–	127	223	–
40	393	+	84	303	+	128	222	–
41	388	+–	85	301	–	129	222	–
42	385	+	86	299	+	130	222	+
43	385	+	87	298	–	131	221	+
44	383	–	88	297	–	132	219	–

Table 4.1 (cont'd)

Sample No.	SCC x 10^3/mL	PCR Results	Sample No.	SCC x 10^3/mL	PCR Results	Sample No.	SCC x 10^3/mL	PCR Results
133	216	-	153	186	+	173	144	-
134	215	-	154	183	+	174	138	+
135	214	-	155	183	+	175	134	-
136	212	-	156	182	+	176	133	-
137	208	-	157	179	-	177	133	+
138	208	-	158	179	-	178	130	+
139	207	-	159	179	-	179	129	-
140	205	+	160	177	-	180	127	-
141	204	-	161	176	-	181	126	+
142	202	-	162	175	-	182	120	-
143	202	-	163	173	-	183	120	-
144	201	-	164	172	-	184	113	-
145	198	+	165	169	-	185	112	-
146	196	-	166	169	-	186	102	-
147	195	-	167	168	+			
148	194	-	168	160	-			
149	194	+	169	152	+			
150	194	-	170	150	-			
151	190	-	171	149	-			
152	188	-	172	146	-			

Table 4.2 Somatic cell count and MB-PCR Results of 165 Bulk Milk samples from Victoria.

Sample No.	SCC x 10^3/mL	PCR Result	Sample No.	SCC x 10^3/mL	PCR Result	Sample No.	SCC x 10^3/mL	PCR Result
1	1,250	+	46	428	–	91	323	–
2	1,027	–	47	425	+	92	314	+
3	1,019	–	48	425	+	93	312	–
4	985	+	49	425	+	94	311	–
5	800	+	50	424	+	95	310	–
6	772	+	51	423	–	96	300	+
7	769	+	52	414	+	97	294	+
8	748	+	53	411	+	98	292	+
9	703	+	54	410	+	99	291	+
10	671	+	55	406	–	100	289	+
11	666	+	56	405	–	101	288	+
12	654	+	57	403	–	102	287	–
13	646	+	58	402	+	103	286	+
14	643	+	59	397	+	104	284	+
15	615	+	60	396	+	105	283	+
16	611	+	61	394	+	106	280	+
17	602	+	62	394	+	107	276	+
18	599	–	63	393	+	108	271	+
19	598	–	64	393	+	109	271	+
20	573	–	65	388	–	110	265	–
21	569	+	66	387	+	111	263	+
22	546	+	67	383	+	112	260	–
23	536	+	68	383	+	113	257	+
24	533	+	69	382	–	114	255	+

Table 4.2 (cont'd)

Sample No.	SCC x 10^3/mL	PCR Result	Sample No.	SCC x 10^3/mL	PCR Result	Sample No.	SCC x 10^3/mL	PCR Result
25	524	+	70	381	–	115	254	+
26	514	+	71	379	–	116	253	+
27	513	+	72	376	–	117	252	+
28	507	+	73	375	–	118	251	–
29	500	+	74	370	+	119	251	+
30	496	+	75	370	+	120	249	–
31	491	+	76	363	+	121	245	+
32	490	+	77	359	+	122	244	+
33	486	+	78	357	–	123	243	+
34	478	+	79	353	–	124	241	–
35	477	+	80	351	–	125	240	–
36	464	+	81	348	+	126	239	+
37	460	+	82	345	+	127	236	+
38	450	+	83	343	–	128	236	+
39	450	+	84	339	–	129	236	–
40	441	+	85	336	+	130	229	–
41	439	+	86	335	+	131	227	–
42	438	–	87	334	+	132	226	+
43	435	–	88	334	+	133	225	–
44	434	+	89	328	+	134	221	–
45	433	+	90	325	+	135	217	–

Table 4.2 (cont'd)

Sample No.	SCC x 10^3/mL	PCR Result	Sample No.	SCC x 10^3/mL	PCR Result
136	215	+	151	172	–
137	213	+	152	168	–
138	210	–	153	159	+
139	202	–	154	156	+
140	199	–	155	154	–
141	196	–	156	147	–
142	195	–	157	146	–
143	192	–	158	141	–
144	191	–	159	139	+
145	191	–	160	138	–
146	189	–	161	122	–
147	185	+	162	110	–
148	181	–	163	104	–
149	179	+	164	103	–
150	177	+	165	59	–

APPENDIX 5

Table 5.1 Somatic cell count and PCR results of 92 composite milk samples from individual cows from the Atherton Tableland.

Cow No.	SCC x 10^3/mL	PCR Results	Cow No.	SCC x 10^3/mL	PCR Results
1	7410	+	47	244	+
2	7065	+	48	238	–
3	6717	+	49	236	–
4	6392	+	50	228	+
5	5818	+	51	228	+
6	5445	+	52	222	+
7	4498	+	53	208	+
8	3711	+	54	202	+
9	3097	+	55	195	–
10	2861	+	56	188	–
11	2823	+	57	185	–
12	2500	+	58	182	+
13	2391	+	59	181	+
14	2282	+	60	172	–
15	2188	+	61	166	+
16	1622	+	62	165	+
17	1495	+	63	163	–
18	1488	+	64	156	+
19	1406	+	65	136	+
20	1321	+	66	129	–
21	1081	–	67	129	+
22	976	+	68	128	–
23	956	–	69	127	–
24	714	–	70	121	+
25	646	–	71	121	–
26	619	+	72	113	+
27	609	+	73	112	+
28	584	+	74	104	+
29	528	+	75	79	–
30	498	+	76	76	–
31	456	+	77	75	–
32	451	+	78	72	–
33	442	+	79	71	–
34	433	–	80	70	+
35	407	–	81	67	+
36	386	+	82	58	+
37	381	–	83	53	+
38	371	+	84	49	–

Table 5.1 (con't)

Cow No.	SCC x 10^3/mL	PCR Results	Cow No.	SCC x 10^3/mL	PCR Results
39	368	+	85	46	–
40	351	+	86	46	–
41	333	+	87	42	+
42	328	+	88	41	+
43	305	+	89	34	–
44	300	–	90	32	–
45	290	+	91	22	+
46	265	–	92	14	+

APPENDIX 6.1

Table 6.1 Quarter milk samples from cows with persistently high somatic cell count. These samples were submitted to the Oonoonba Veterinary Laboratory for culture for mastitis pathogens.

Accession No: 95-50235 **Date: 05.12.95**

Animal No.	Quarter No.	Culture Result	MB-PCR Result
1	1.1	No aerobic growth	–
	1.2	No aerobic growth	–
	1.3	No aerobic growth	–
	1.4	*Staphylococcus aureus*	+
2	2.1	No aerobic growth	+
	2.2	No aerobic growth	+
	2.3	*Staphylococcus aureus*	+
3	3.1	No aerobic growth	–
	3.2	*Serratia marcescens*	–
	3.3	*Serratia marcescens*	–
	3.4	No aerobic growth	–
4	4.1	No aerobic growth	–
	4.2	*Staphylococcus aureus*	+
	4.3	*Staphylococcus aureus*	–
	4.4	*Staphylococcus aureus*	–
5	5.1	*Klebsiella oxytoca and Serratia marcescens*	–
	5.2	No aerobic growth	–
	5.3	No aerobic growth	+
6	6.1	*Staphylococcus aureus*	–
	6.2	No aerobic growth	–
	6.3	*Staphylococcus aureus*	–
7	7.1	*Staphylococcus aureus*	+
	7.2	*Streptococcus dysgalactiae*	–
	7.3	*Staphylococcus aureus*	+
	7.4	No aerobic growth	+
8	8.1	No aerobic growth	–
	8.2	No aerobic growth	–
	8.3	No aerobic growth	–
	8.4	No aerobic growth	–
9	9.1	*Staphylococcus aureus*	–
	9.2	No aerobic growth	–
	9.3	*Streptococcus dysgalactiae*	–
	9.4	*Streptococcus dysgalactiae*	–

APPENDIX 6.2

Table 6.2 Quarter milk samples from cows with persistently high somatic cell count. These samples were submitted to the Oonoonba Veterinary Laboratory for culture for mastitis pathogens.

Accession No: 95-50394 **Date: 05.12.95**

Animal No.	Quarter No.	Culture Result	MB-PCR Result
1	1.1	No aerobic growth	+
	1.2	No aerobic growth	+
	1.3	No aerobic growth	–
	1.4	*Serratia marcescens*	–
2	2.1	*Micrococcus* spp.	+
	2.2	No aerobic growth	–
	2.3	No aerobic growth	–
	2.4	No aerobic growth	–
3	3.1	No aerobic growth	–
	3.2	No aerobic growth	+
	3.3	No aerobic growth	+
	3.4	No aerobic growth	+
4	4.1	No aerobic growth	–
	4.2	No aerobic growth	–
	4.3	*Micrococcus* spp.	–
	4.4	No aerobic growth	–
5	5.1	No aerobic growth	–
	5.2	*Micrococcus* spp.	–
	5.3	No aerobic growth	–
	5.4	No aerobic growth	–
6	6.1	No aerobic growth	–
	6.2	No aerobic growth	–
	6.3	*Micrococos* spp.	–
	6.4	No aerobic growth	+

APPENDIX 6.3

Table 6.3 Quarter milk samples from cows with persistently high somatic cell count. These samples were submitted to the Oonoonba Veterinary Laboratory for culture for mastitis pathogens.

Accession No: 95-46992 **Date: 25.8.95**

Animal No.	Quarter No.	Culture Result	MB-PCR Result
1	1.1	No aerobic growth	–
	1.2	No aerobic growth	–
	1.3	No aerobic growth	–
	1.4	No aerobic growth	–
2	2.1	No aerobic growth	–
	2.2	No aerobic growth	–
	2.3	No aerobic growth	+
	2.4	No aerobic growth	–
3	3.1	*Corynebacterium bovis*	–
	3.2	*Streptococcus*	–
	3.3	*Streptococcus*	–
	3.4	*Streptococcus*	–
4	4.1	No aerobic growth	+
	4.2	No aerobic growth	–
	4.3	No aerobic growth	–
	4.4	No aerobic growth	–
5	5.1	*Streptococcus*	+
	5.2	*Streptococcus*	+
	5.3	No aerobic growth	–
	5.4	No aerobic growth	–
6	6.1	*Corynebacterium bovis*	–
	6.2	*Corynebacterium bovis*	–
	6.3	*Corynebacterium bovis*	+
	6.4	*Corynebacterium bovis*	–
7	7.1	No aerobic growth	–
	7.2	No aerobic growth	–
	7.3	No aerobic growth	+
	7.4	No aerobic growth	–
8	8.1	*Corynebacterium bovis*	–
	8.2	No aerobic growth	–
	8.3	No aerobic growth	–
	8.4	No aerobic growth	–

Table 6.3 (con'td)

Animal No.	Quarter No.	Culture Result	MB-PCR Result
9	9.1	No aerobic growth	–
	9.2	*Corynebacterium bovis*	+
	9.3	No aerobic growth	–
	9.4	No aerobic growth	–
10	10.1	No aerobic growth	–
	10.2	No aerobic growth	+
	10.3	No aerobic growth	–
	10.4	No aerobic growth	–

APPENDIX 6.4

Table 6.4 Quarter milk samples from cows with persistently high somatic cell count. These samples were submitted to the Oonoonba Veterinary laboratory for culture for mastitis pathogens.

Accession No: 95-47233 **Date: 01.09.95**

Animals No.	Quarter No.	Culture Result	MB-PCR result
1	1.1	No aerobic growth	+
	1.2	No aerobic growth	+
	1.3	No aerobic growth	+
	1.4	*Staphylococcus spp.*	+
2	2.1	*Staphylococcus spp.*	–
	2.2	*Staphylococcus spp.*	–
	2.3	*Staphylococcus spp.*	–
	2.4	No aerobic growth	–
3	3.1	No aerobic growth	–
	3.2	No aerobic growth	–
	3.3	*Staphylococcus spp.*	–
	3.4	No aerobic growth	–
4	4.1	No aerobic growth	–
	4.2	No aerobic growth	+
	4.3	No aerobic growth	+
	4.4	*Staphylococcus spp.*	–
5	5.1	No aerobic growth	–
	5.2	No aerobic growth	–
	5.3	No aerobic growth	–
	5.4	No aerobic growth	+
6	6.1	*Serratia marcescens*	+
	6.2	No aerobic growth	+
	6.3	No aerobic growth	+
	6.4	No aerobic growth	–
7	7.1	No aerobic growth	–
	7.2	No aerobic growth	–
	7.3	No aerobic growth	–
	7.4	No aerobic growth	–
8	8.1	No aerobic growth	–
	8.2	*Enterococci agglomerans*	+
	8.3	*Serratia marcescens*	+
	8.4	No aerobic growth	+

Animals No.	Quarter No.	Culture Result	MB-PCR result
9	9.1	No aerobic growth	–
	9.2	*Serratia marcescens*	+
	9.3	No aerobic growth	–
	9.4	No aerobic growth	–
10	10.1	No aerobic growth	+
	10.2	No aerobic growth	–
	10.3	*Pseudomonas spp.*	+
	10.4	No aerobic growth	+

APPENDIX 6.5

Table 6.5 Quarter milk samples from cows with persistently high somatic cell count. These samples were submitted to the Oonoonba Veterinary Laboratory for culture for mastitis pathogens.

Accession No: 96-40786 **Date: 07.02.96**

Animal No.	Quarter No.	Culture Result	MB-PCR Result
1	1.1	No aerobic growth	–
	1.2	*Klebsiella oxytoca*	+
	1.3	*Bacillus* spp.	–
	1.4	*Bacillus* spp.	+
2	2.1	*Staphylococcus aureus*	–
	2.2	*Serratia marcescens*	+
	2.3	*Enterococci cloacae*	–
		Staphylococcus aureus	
	2.4	*Serratia marcescens*	–
3	3.1	*Staphylococcus* spp.	+
	3.2	No aerobic growth	–
	3.3	*Enterococci cloacae*	+
	3.4	*Staphylococcus aureus*	–
4	4.1	No aerobic growth	–
	4.2	*Enterococci cloacae*	+
	4.3	*Micrococus* spp.	–
	4.4	*Bacillus* spp.	–
5	5.1	No aerobic growth	+
	5.2	No aerobic growth	–
	5.3	No aerobic growth	–
	5.4	No aerobic growth	+
6	6.1	*Klebsiella oxytoca*	+
	6.2	No aerobic growth	–
	6.3	No aerobic growth	–
	6.4	No aerobic growth	+
7	7.1	No aerobic growth	+
	7.2	*Klebsiella oxytoca*	+
		Micrococcus spp.	
	7.3	*Enterococci agglomerans*	+
	7.4	*Enterococci cloacae*	–

Animal No.	Quarter No.	Culture Result	MB-PCR Result
8	8.1	No aerobic growth	+
	8.2	*Enterococci agglomerans*	+
	8.3	No aerobic growth	–
	8.4	*Klebsiella pneumoniae*	–
9	9.1	No aerobic growth	–
	9.2	No aerobic growth	+
	9.3	*Staphylococcus aureus*	+
	9.4	No aerobic growth	+
10	10.1	No aerobic growth	–
	10.2	No aerobic growth	+
	10.3	No aerobic growth	–
	10.4	No aerobic growth	–

APPENDIX 6.6

Table 6.6 Quarter milk samples from cows with persistently high somatic cell count. These samples were submitted to the Oonoonba Veterinary Laboratory for culture for mastitis pathogens.

Accession No: 96-41894 **Date: 20.3.96**

Animal No.	Quarter No.	Culture Result	PCR Result
1	1.1	No aerobic growth	–
	1.2	No aerobic growth	–
	1.3	No aerobic growth	+
	1.4	No aerobic growth	+
2	2.1	No aerobic growth	–
	2.2	*Bacillus* spp.	–
	2.3	*Bacillus* spp.	–
	2.4	*Corynebacterium bovis*	+
3	3.1	*Corynebacterium bovis*	+
	3.2	*Citrobacter.freundii* and *Micrococcus* spp.	–
	3.3	No aerobic growth	+
	3.4	No aerobic growth	+
4	4.1	*Staphylococcus aureus*	+
	4.2	*Staphylococcus aureus* and *Streptococcus uberis*	+
	4.3	*Staphylococcus aureus*	+
	4.4	*Streptococcus uberis*	+
5	5.1	No aerobic growth	+
	5.2	No aerobic growth	–
	5.3	*Staphylococcus aureus*	–
	5.4	No aerobic growth	–
6	6.1	No aerobic growth	–
	6.2	No aerobic growth	+
	6.3	No aerobic growth	–
	6.4	No aerobic growth	–
7	7.1	*Neisseria morganii*	+

APPENDIX 7.1

Table 7.1 Raw data of PCR and culture results of quarter milk samples from Farm 1 at Atherton Tableland and their association with serology, as detected by blocking ELISA.

Date: 05.09.1996

Cow No.	Quarter No.	*M. bovis* PCR results	*Mycoplasma* Culture	Blocking ELISA Inhibition %
194	1	+	+	
	2	+	+	46.2
	3	–	–	
	blind	N/A	N/A	
244	1	+	–	
	2	–	–	65.6
	blind	N/A	N/A	
	4	+	–	
52	1	–	–	
	2	–	–	57.4
	3	+	–	
	4	–	–	
1	1	+	–	
	2	–	–	55.8
	3	–	–	
	4	–	–	
249	1	–	–	
	2	–	–	62.4
	3	+	–	
	4	–	–	
79	1	–	–	
	2	–	–	71.1
	3	+	–	
	4	–	–	
206	1	–	–	
	2	–	–	51.0
	3	–	–	
	4	–	–	
236	1	–	–	
	2	–	–	51.0
	3	+	–	
	4	+	–	
23	1	–	–	
	2	–	–	63.4
	3	–	–	
	4	+	–	

Table 7.1 (cont'd)

Cow No.	Quarter No.	*M. bovis* PCR results	*Mycoplasma* Culture	Blocking ELISA Inhibition %
49	1	–	–	
	2	–	–	56.0
	3	–	–	
	4	+	–	
61	1	+	–	
	blind	N/A	N/A	58.7
	3	+	–	
	4	+	–	
75	1	–	–	
	2	+	–	61.0
0	3	–	–	
	4	–	–	
11	1	–	–	
	2	+	–	54.3
	3	–	–	
	4	–	–	
259	1	–	–	
	2	+	–	68.3
	3	+	–	
	4	+	–	

N/A: Not applicable

APPENDIX 7.2

Table 7.2 Raw data of PCR and culture results of quarter milk samples from Farm 2 at Atherton Tableland, their association with serology as detected by blocking ELISA, and prevalence of respiratory infection.

Date: 05.09.1996

Animal No.	Quarter No.	*M. bovis* PCR results	*Mycoplasma* culture	Nasal swab PCR	Blocking ELISA Inhibition %
250	1	+	–		
	2	–	–	+	58.5
	3	+	–		
	4	–	–		
313	1	+	–		
	2	–	–	+	54.2
	3	–	–		
	4	–	–		
227	1	–	–		
	2	+	–	+	53.5
	3	–	–		
	4	+	–		
145	1	–	Not tested		
	2	–	Not tested	–	52.9
	3	–	Not tested		
	4	–	Not tested		

Table 7.2 (cont'd)

Animal No.	Quarter No.	*M. bovis* PCR results	*Mycoplasma* culture	Nasal swab PCR	Blocking ELISA Inhibition %
153	1	–	–		
	2	+	–	+	67.0
	3	+	–		
	4	–	–		
257	1	–	Not tested		
	2	–	Not tested	–	51.0
	3	–	Not tested		
	4	–	Not tested		
253	1	+	–		
	2	–	–	+	48.9
	3	–	–		
	4	–	–		
306	1	+	–		
	2	–	–	+	52.0
	3	–	–		
	4	+	–		
833	1	–	Not tested		
	2	–	Not tested	–	44.5
	3	–	Not tested		
	4	–	Not tested		
152	1	–	Not tested		
	2	–	Not tested	–	36.7
	3	–	Not tested		

Table 7.2 (cont'd)

Animal No.	Quarter No.	*M. bovis* PCR results	*Mycoplasma* culture	Nasal swab PCR	Blocking ELISA Inhibition %
	4	–	Not tested		

Table 7.2 (cont'd)

Animal No.	Quarter No.	*M. bovis* PCR results	*Mycoplasma* culture	Nasal swab PCR	Blocking ELISA Inhibition %
934	1	+	–		
	2	–	–	+	72.2
	3	–	–		
	4	–	–		
228	1	–	–		
	2	–	–	+	52.2
	3	–	–		
	4	+	–		
926	1	–	–		
	2	–	–	+	57.9
	3	+	–		
	4	+	–		
347	1	+	–		
	2	–	–	+	51.0
	3	–	–		
	4	–	–		
201	1	–	Not tested		
	2	–	Not tested	–	48.3
	3	–	Not tested		
	4	–	Not tested		