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The Diagnosis of an Outbreak of *Mycoplasma bovis* Clinical Mastitis in a Multi-farm North Otago Farming Operation

A thesis presented in partial fulfilment of the requirements
for the degree of

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at Massey University, Manawatū, New Zealand.

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Abstract

Mycoplasma bovis (*M. bovis*) causes a multitude of disease syndromes in dairy cattle including clinical mastitis (CM), arthritis and pneumonia. The detection in July 2017 of *M. bovis,* for the first time in New Zealand (NZ), on a South Island dairy farm, prompted a national animal disease response. This descriptive study aims to describe the clinical and diagnostic test findings of an outbreak of *M. bovis* CM, on a large multi-farm dairy enterprise where there was a single hypothesised infection source and date. Samples were collected as part of surveillance activities on-farm and at slaughter, together with farmer-selected CM cows, to provide results from real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) tests of bulk tank milk (BTM), individual cow serum ELISA tests, quarter milk samples (QMS), and palatine tonsils qPCR tests. Post-mortem sampling of the mammary glands of *M. bovis* CM cases was also performed. Positive BTM PCR, supported by BTM ELISA, confirmed infection in two of the four dairy herds in the enterprise and herd-level serology (serum ELISA) confirmed infection in a third herd. There was a common clinical presentation in infected herds of an unusually high incidence of apparent treatment failure (ATF) of non-systemically ill, multiple quarter CM cases, from some of which *M. bovis* was detected. Individual CM cases were found in the main to be QMS *M. bovis* qPCR positive, serum ELISA positive and palatine tonsil qPCR positive. In approximately 70% of *M. bovis* CM cases, *M. bovis* was found to be the sole pathogen. A smoothed function model between serum ELISA and time from first diagnosis of CM, from which *M. bovis* was detected, predicted that the average interval between clinical diagnosis and a serum ELISA test positive result was five days. The higher observed agreement between the serum ELISA and palatine tonsil qPCR, was for *M. bovis* CM cows sampled on-farm compared with cows sampled at slaughter. Gross lesions of fibrosis, caseous necrosis and cystic dilation in the udders of *M. bovis* positive CM cows were seen together with granulomatous and suppurative inflammatory patterns histologically. High immunoreactivity in immunohistochemistry for the *M. bovis* antigen was also present. From the key diagnostic test findings, *M. bovis* was likely to have been one of several pathogens which caused individual cases of CM on the farming enterprise, and in many cases may have been the sole cause of CM cases. The results of this study can raise awareness of and provide information to aid dairy farmers and veterinarians determine if *M. bovis* has a role in CM outbreaks with unexpectedly increased numbers of treatment failures and can inform the regulatory response for surveillance and testing of herds and individual cattle for *M. bovis.*

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Glossary

List of Abbreviations

Ab – Antibody

ACR – Automatic cup removers

AHL – Animal Health Laboratory

AI – Artificial insemination

AFLP – Amplified fragment length polymorphism

AFO – Acid fast organism

AP – Apparent prevalence

ATF – Apparent treatment failure

ATS – Apparent treatment success

BHV-1 – Bovine herpes virus 1

BLAST - Basic Local Alignment Search Tool

BMSCC – Bulk milk somatic cell count

BMT – Bulk milk testing

BPI-3 – Bovine Parainfluenza-3 virus

BRDC – Bovine respiratory disease complex

BRSv – Bovine respiratory syncytial virus

BTM – Bulk tank milk

BVDV – Bovine diarrhoea virus

cfu – colony forming units

CM – Clinical Mastitis

CMI – Cell mediated immunity

CMS – Composite milk sample

CNS – Coagulase Negative Staphylococcus

CT – Cycle threshold

DDGE – Denaturing gradient gel electrophoresis

DNA – Deoxyribonucleic acid

EBL – Embryonic bovine lung

edf – Estimated degrees of freedom

ELISA – Enzyme-linked immunosorbent assay

GAM – Generalised additive model

HE – Haematoxylin and eosin

ICR – In calf rate

Ig - Immunoglobulin

IHA – Indirect Haemagglutination

IHC – Immunohistochemistry

i.m - Intramuscular

IMI – Intramammary infection

IP – Incubation period

ISCC – Individual somatic cell count

KPI – Key performance indicators

LCA – Latent class analysis

LIC – Livestock Improvement Corporation

LID – Lifetime identification

LOSM – Lower order sharemilker

MALDI-TOF - matrix-assisted laser desorption/ionisation – time of flight mass spectrometry

MbAD – *Mycoplasma bovis*-associated disease

MIC – Minimum inhibitory concentration

MLST – Multi-locus sequence typing

MLVA – Multiple locus variable number tandem repeat analysis

MPI – Ministry for Primary Industries

NADH - Nicotinamide Adenine Dinucleotide Hydrogen

NAIT – National animal identification and tracing

NCBI – National Centre for biotechnology information

NEB – Negative energy balance

NEFA – Non-esterified fatty acid

NGS – Next generation sequencing

NO – North Otago

NZ – New Zealand

OAD – Once a day milking

ODC – Optical density coefficient

PBMC – Peripheral blood mononuclear cells

PCR – Polymerase chain reaction

PFGE – Pulse-field gel electrophoresis

PI – Persistently infected

PM – post-mortem

QMS – Quarter milk sample

qPCR – Real-time PCR

RAPD – Random amplified polymorphic DNA

RFID – Radio-frequency identification

RMT – Rapid mastitis test

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

rRNA – Ribosomal RNA

s.c – Subcutaneous

SCM – Subclinical mastitis

S/P – Sample to positive

Se – Sensitivity

Sp – Specificity

spp. - Species

ST – Sequence type

TAD – Twice a day milking

TMR – Total Mixed Ration

URT – Upper respiratory tract

Vsps – Variable surface lipoproteins

WGS – Whole genome sequence

WHP – Withholding period

WHT – Withholding time

Chapter 1 Introduction

1.1 Introduction

Mycoplasma bovis is a highly contagious pathogen of cattle and is known to cause significant economic losses and animal welfare impacts worldwide (Nicholas, 2011). There are numerous clinical manifestations of *M. bovis* in a herd, including mastitis, arthritis and pneumonia in cattle of all ages (Maunsell et al., 2011; Nicholas & Ayling, 2003), and otitis media in young calves (Maunsell et al., 2012). The presence of asymptomatic carrier animals in an infected herd, together with the poor diagnostic test sensitivity of samples from individual animals, make *M. bovis* elimination from a herd very difficult (Gille et al., 2018; Maunsell et al., 2011). Additionally, there are still large gaps in our understanding of the epidemiology and pathophysiology of *M. bovis* disease (Calcutt et al., 2018) that make control of this pathogen difficult (Fox et al., 2005).

The clinical signs of mycoplasmal mastitis and specifically *M. bovis* clinical mastitis (CM) are considered nonspecific. The classical presentation is a mastitis which progresses to a chronic multi-quarter mastitis, with a marked drop in milk production and milk quality, but systemic clinical signs are rarely reported. Clinical cases are unresponsiveness to antimicrobial therapy, and the mastitis may become purulent in nature and progress to agalactia (Nicholas et al., 2016; Pothmann et al., 2015; Radaelli et al., 2011). Additionally, the epidemiological characteristics of *M. bovis* infection at the herd-level are reported to be related to management factors such as herd size, introduction of stock to a farm and animal replacement policies (McCluskey et al., 2003; Punyapornwithaya et al., 2010). However, the main body of work on the clinical manifestations of *M. bovis* has been reported in European farming system (Petersen et al., 2018; Vähänikkilä et al., 2019), which are quite dissimilar to those in New Zealand (NZ) which may therefore impact clinical presentation.

The July 2017 detection of *M. bovis* in a NZ South Island dairy farm prompted a national response. In NZ, the Ministry for Primary Industries (Manatu Ahu Matua) (MPI), is the competent authority which responds to incursions of Unwanted and New Organisms, in accordance with the Biosecurity Act 1993 (Government, 1993). In May 2018, the NZ Government, together with the dairy and beef industries, made the decision to eradicate *M. bovis* from NZ (Ministry for Primary Industries, 2019b; New Zealand Government, 2018).

At the time of undertaking this research, there was only one brief report of *M. bovis* clinical disease in a NZ dairy herd, from the index case (Hay, 2018). Additionally, the management effects on the epidemiology of *M. bovis* and the use of different diagnostic tests in other countries limit the relevance of the descriptions of outbreaks in other countries to the NZ farmer and veterinarian. Therefore, there

is a need to describe an outbreak of *M. bovis* CM in the NZ setting. This thesis investigates an outbreak of *M. bovis* CM on a multi-farm dairy operation and reports, in detail, herd-, cow- and quarter-level epidemiological findings, gross and microscopic lesions in infected mammary glands, as well as considering the agreement of two diagnostic tests used in the situation.

The three tenets of a veterinary diagnosis of a disease are firstly identify, the specific cause, secondly identify the abnormality of structure or function that the causative agent has produced and which is detrimental to normal body structure or function, and thirdly, identify the clinical manifestation of the causative agent (Radostits et al., 2000). Hazelton et al. (2018) considers a clinical diagnosis of *M. bovis*-associated disease (MbAD) to be based on the clinical presentation with confirmation by microbiological culture or polymerase chain reaction (PCR) of the milk, or other tissues. Given the clinical signs of infection associated with *M. bovis* are non-specific, Wawegama and Browning (2017) suggest a tentative diagnosis is reached when clinical disease is present, with a combination of clinical signs, postmortem (PM) findings, histopathology and immunohistochemistry (IHC). However, further testing is required to determine the prevalence of disease at the herd level. González and Wilson (2003) reminded the veterinary practitioner to keep an open mind diagnosing intramammary infections (IMI) on dairy farms, as the variations in the pathogenicity of different strains of *M. bovis,* of animal susceptibility, and of farming practices on the dairy farm are key determinants for the presentation of mycoplasmal mastitis.

This descriptive study includes both case and prospective cohort study methods to describe key epidemiological, clinical and pathological findings which enabled the diagnosis of *M. bovis* CM to be made in NZ, across a multi-farm dairy operation, with a single hypothesized infection source and date. While the findings in this case study will offer an understanding of the use of a range of diagnostic tests, applied both at a herd- and cow-level, it is imperative for the NZ dairy industry that the diagnosis of *M. bovis* CM at a cow-level can be made by veterinarians in the field. This would allow the implementation of effective control measures in a timely way to limit further spread of infection within and between farms.

The three objectives of this thesis are to:

1. Provide an epidemiological description of the diagnostic test findings and clinical manifestations of a case study of an outbreak of *M. bovis* CM in NZ. This will be reported on a herd-, cow-, and quarter-level with the expectation to provide NZ-based data for a dairy clinician to make a diagnosis of *M. bovis* CM. This will be reported as the “Outbreak Investigation”.

2. Provide a comparison on how well the serum enzyme-linked immunosorbent assay (ELISA) sample to positive (SP) ratio agreed with tonsil swab real-time PCR (qPCR) in both clinical and non-

clinical cows. The tonsil qPCR, as a parallel diagnostic test, could be used as part of an eradication program, or wider surveillance program, to confirm the clinical diagnosis of *M. bovis.* This will be reported as the "Agreement Study".

3. Present the patterns of the gross lesions found in cases of *M. bovis* CM. This description will enable the dairy clinician to use gross pathology (and associated tests) as additional diagnostic tools to recognize *M. bovis*-associated lesions in the mammary gland, as different morphological patterns are currently unreported. This will be reported as "Pathological Findings – Morphological patterns of mammary gland lesions in dairy cows with *M. bovis* CM".

1.2 Format of Thesis

Chapter 2 Literature Review summarises the scientific literature of *M. bovis*. This review will primarily detail our current knowledge of the epidemiology and clinical manifestations of *M. bovis*, the diagnostic tests used in the diagnosis of *M. bovis*, the pathology of M. *bovis* CM and finally describe the control and eradication strategies for *M. bovis.*

Chapter 3 Material and Methods outlines the background to the case study, collection of samples, laboratory analysis, data sources used and statistical analysis of data.

Chapter 4 Results reports the findings of the three main study objectives.

Chapter 5 Discussion considers the findings of this study in the context of other relevant literature and draws conclusions on each of the three objectives. These conclusions will aid the veterinary diagnosis of *M. bovis* CM. The limitations of this study, and their possible impact, will also be highlighted. Important directions for future research are considered.

Chapter 2 Literature Review

2.1 Introduction

This review details *M. bovis* as a pathogen, followed by the epidemiology of *M. bovis*, specifically pathogenicity, transmission, colonisation, shedding, prevalence, and risk factors. The international literature describing the clinical manifestations of *M. bovis*, with emphasis on *M. bovis* mastitis and associated pathology, together with the diagnostic tests used in the diagnosis of *M. bovis* are examined. Finally, the control and eradication of *M. bovis* is considered.

2.2 *Mycoplasma bovis* – The Pathogen

2.2.1 Microbiology

The first recorded CM outbreak in a dairy herd due to the species now called *M. bovis* was in Connecticut, USA in 1961 (Hale et al., 1962). The bacterium has had several reclassifications over the decades, from *Mycoplasma agalactiae* var *bovis*, to *Mycoplasma bovimastitidis* in 1967 (Jain et al., 1967) then *Mycoplasma agalactiae* subspecies *bovis* in 1970 (Freundt & Edward, 1971) and finally in 1976 after further microbiological observations, these strains were named as a new species, *M. bovis* (Askaa, 1976). Seminal work on *M. bovis* mastitis which was reported during this period of taxonomic change (Jain et al., 1969; Jasper & Al Aubaidi, 1974) and mainly reported on experimentally induced disease, still offers some of the more detailed reports of disease progression.

The Genus *Mycoplasma* belong to the Class Mollicutes and are a class of bacteria distinguished by the absence of a cell wall. *Mycoplasma bovis* is small and pleomorphic; with a small genome size of 1,080 kilobase pairs (kbps) and low G+C ratio of 27.8-32.9 mol%. The bacterium has complex nutritional requirements, lacks the tricarboxylic acid cycle (TCA), relies on the host for external sources of lipids, amino acids, nucleic acid precursors and is unable to ferment glucose (Hermann R, 1992; Khan et al., 2005). *Mycoplasma bovis* can survive at 4°C for nearly 2 months in sponges and milk, and over 2 weeks in water; however at higher temperatures, survival drops considerably (Ruffo et al., 1974; Ruffo et al., 1969). Without a cell wall, the cell membrane is directly exposed to the host environment and the bacterium is vulnerable to osmotic shock (Bürki et al., 2015; Kumar et al., 2014) and resistant to antimicrobials of the beta-lactam family, which inhibit the synthesis of peptidoglycan (Rosenbusch, 1994).

2.2.2 Mycoplasma Species

There are over 100 species of mycoplasmas (Razin et al., 1998). *Mycoplasma bovis* is the most common cause of mycoplasmal mastitis in cows (Ayling et al., 2004; Bushnell, 1984; González & Wilson, 2003). *Mycoplasma alkalescens*, *Mycoplasma canadense*, *Mycoplasma californicum*, and *Mycoplasma bovigenitalium* together with *M. bovis* are considered the more common *Mycoplasma* spp. which cause mastitis (González & Wilson, 2003). Other species associated with mastitis outbreaks include *Mycoplasma* bovine group 7, now named *Mycoplasma leachii* (Hum et al., 2000), *Mycoplasma bovirhinis* (Hirose et al., 2001), and *Mycoplasma dispar* (Jasper, 1981a). *Mycoplasma canis*, *Mycoplasma gallinarium* and *Mycoplasma bovoculi* have been isolated from milk samples, but their role in bovine mastitis is not clear (Ayling et al., 2004). Differentiation of species by culture can be challenging as they possess similar morphology, cultural and biochemical characteristics (Fox et al., 2005; Kumar et al., 2014; Parker et al., 2018). While *M. bovis* and *Mycoplasma agalactiae* share a number of related proteins, epitopes, and antigenic determinants, they can be diagnostically differentiated by DNA-based detection methods more easily, as the PCR assays target different genomic regions (Bashiruddin et al., 2005).

Two species of mycoplasma have previously been reported in NZ dairy cattle, where the key clinical syndrome has been outbreaks of unresponsive mastitis (Pharo, 2018). In 1969, a member of the bovine mycoplasma group 8, now renamed *M. alkalescens* was diagnosed in Northland (Brookbanks E et al., 1969), and in 1983 in the Waikato, *M. dispar* was diagnosed in an outbreak of dry cow mastitis (Hodges R et al., 1983). Both of these species have been described as infrequent and sporadic cause of mastitis, with low transmission rates (Rosenbusch, 2005). Several other mycoplasmal diseases have been reported in cattle in NZ. Diseases include regenerative anaemia and haemoglobuinuria in a cow where haemotropic mycoplasmas (M. *wenyonii*, Candidatus M. haemobos) were detected by PCR (McFadden et al., 2016), and polyarthritis in a cow where *Mycoplasma mycoides mycoides* large colony (MmmLC) was demonstrated on both PCR and culture of the joint tissues (Johnstone & King, 2003).

2.2.3 The Use of Molecular Techniques for Typing Mycoplasma Strains

Bacteria can be classified firstly into genus e.g. *Mycoplasma*, then secondly, species e.g. *M. bovis*, and the third level of classification is strain classification e.g. Strain PG45, Strain M590 (Aebi et al., 2012). While the bacteriological terms strain and clone have specific scientific definitions, some scientific papers, do not define them well (Dijkshoorn et al., 2000), with some strain studies for *M. bovis* interchanging the terms (Aebi et al., 2012).

Strain typing has many uses especially for epidemiological analysis. These include firstly, tracing the region or country of origin of an *M. bovis* outbreak, especially where movement of imported animals are suspected (Ayling et al., 2004), and secondly use in epidemiological studies of *M. bovis* and *M. californicum* for between-herd, within-herd and also within-cow studies (Hata et al., 2014). Thirdly, the study of certain aspects of mycoplasma diseases i.e. improving the ability to classify and characterize mycoplasma strains, assessing the genetic diversity of mycoplasmal populations, strain virulence, disease outcomes and importantly, added to our knowledge of internal dissemination of *M. bovis* within the animal (Biddle et al., 2005).

Isolates of *M. bovis* can be genetically characterized using a number of different methods, the details of which are outside the scope of this review. The six common methods are random amplified polymorphic DNA (RAPD), pulse-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) (McAuliffe et al., 2004), insertion sequence fingerprinting (IS) (Miles et al., 2005), multiple locus variable number tandem repeat analysis (MLVA) (Pinho et al., 2012), and multi-locus sequence typing (MLST) (Rosales et al., 2015).

Seminal work by Biddle and co-workers, showed in a mastitis outbreak on one farm, that PFGE patterns of mammary gland isolates were often (44/70) identical to isolates collected from multiple other body sites (Biddle et al., 2005). This finding suggested that there is potential for haematogenous spread of mycoplasmas (Biddle et al., 2005). An Idaho, USA study looking at *M. bovis* mastitis strains also using the PGFE method, found while four strains of *M. bovis* were isolated from different body sites, only one strain, Strain O, caused CM. It was proposed that a virulence factor present in Strain O enabled it to cause CM (Punyapornwithaya et al., 2010). This finding conflicts with other reports, where it was considered that all strains, regardless where in the body they were isolated from, can cause CM (Pfützner & Sachse, 1996).

Danish work (Kusiluka, Kokotovic, et al., 2000) looked at over 40 field isolates of *M. bovis* over a 17-year period and found significant genetic homogeneity. They employed the ALFP method and their findings were consistent with the American work of Biddle et al. (2005). In one cow, they found indistinguishable genetic patterns from *M. bovis* isolates from the nose, lung, and milk, which

suggested the pathogen spread internally. This study also demonstrated the AFLP technique could be used for both discrimination of M. *bovis* strains and genomic fingerprinting. In contrast, an investigation into the prevalence of *M. bovis* in pneumonic lungs in Danish cattle, using PFGE, found the 11 *M. bovis* field isolates from nine different farms, showed different profiles, except for two isolates from the same farm (Kusiluka, Ojeniyi, et al., 2000).

An increase in *M. bovis* mastitis outbreaks in Switzerland initiated a large strain study, with over 1400 samples collected from 19 herds, to determine if one clone or strain had caused these outbreaks. Insertion sequencing techniques showed *M. bovis* strains diverged between herds, and strains were mostly herd specific. The outbreaks were deemed to be caused by numerous strains and not the introduction of a sole new strain or clone (Aebi et al., 2012).

Multiple locus variable number tandem repeat analysis and MLST, a powerful DNA-typing tool for evaluating intraspecies genetic relatedness, are also used for genotyping isolates of *M. bovis* (Sulyok et al., 2014). A large number of *M. bovis* isolates, 137 collected from 12 countries, from both clinically infected and healthy cows, were analyzed by MLST. The isolates fell into two population clusters that were distinct. This finding was in agreement with the hypothesis that geographical independent evolution of *M. bovis* occurs when it is introduced into a new country (Rosales et al., 2015). The MLST method was used to analyze the lineage of a large number of Swiss and Austrian *M. bovis* isolates. Two distinct lineages were described, one for isolates collected since 2007, and the other prior to 2007. Further work is needed to understand if infection with isolates in the 2007 and beyond cluster, lineage 1, leads to more severe outbreaks of mastitis compared to infections with isolates in the prior to 2007 lineage, lineage 2, as there has been an emergence of severe *M. bovis*-associated mastitis cases seen in both countries (Bürki et al., 2016).

The MLST method has been used in the *M. bovis* outbreak in NZ. This method involves PCR amplification followed by DNA sequencing, and measures variations in the DNA sequence of a set of housekeeping genes. Strains are then characterized by unique allelic profiles, which are assigned as a sequence type (ST). Phylogenetic models output based on the *M. bovis* isolates collected in NZ, which compare STs of different clonal complexes, suggest the isolates of *M. bovis* found in NZ all originated from a single strain (Biosecurity New Zealand, 2018b). It has therefore been hypothesized that there has been an incursion of only a single *M. bovis* strain into NZ.

2.3 Epidemiology

The epidemiology of *M. bovis*, including the pathogenicity, transmission, colonisation (or carriage), shedding, prevalence, incidence rates, and risk factors of *M. bovis* is reviewed. While the literature offers consensus is some areas, conjecture and opposing views are raised in others. However, there is agreement that there are still areas of the epidemiology of *M. bovis* that require further investigation.

2.3.1 Pathogenicity

The understanding of the pathogenic characteristics of *M. bovis* and their role in the pathogenesis of disease is still limited, but does appear to be multifactorial (Bürki et al., 2015). *Mycoplasma bovis* has microbial characteristics that facilitate it to both colonise and persist on a number of host mucosal surfaces, and then adapt to the hosts' immune response to persist and cause disease (Lysnyansky et al., 1999). The virulence factors of *M. bovis* to be reviewed are antigenic variation, which includes the presence of variable surface lipoproteins (Vsps) and chromosomal rearrangement, adherence to and internalization into host cells, immunomodulatory characteristics, the production of secondary metabolites, biofilm production and synergistic infections with other pathogens. These virulence factors allow the bacterium to elude defence mechanisms of the host and hence persist within host cells (Bürki et al., 2015). *Mycoplasma bovis* can also colonise several host sites without fulminant disease developing.

2.3.1.1 Antigenic Variation

Variation in the expression of the cell surface antigens is thought to be a form of adaptive variation by the bacteria to the host. An important source of these surface antigens are exposed membrane proteins, which are a group of immuno-dominant Vsps. These Vsps are one of the best-studied pathogenetic mechanisms of *M. bovis*. This family of proteins undergoes high-frequency phase (turning expression ON and OFF) and size variation, which results in the cell gaining and/or losing surface antigens (Bürki et al., 2015). As an example, in the type strain PG45 of *M. bovis*, the vsp-locus, which is an organised chromosomal cluster, is comprised of a family of 13 distinctive, single-copy vsp genes. The deduced proteins are identified as VspA to VspO (Behrens et al., 1994; Lysnyansky et al., 1999). Features like these allow *M. bovis* to evade immune surveillance and facilitates chronic infections (Buchenau et al., 2010). Apart from antigenic variation, Vsps have also been associated with other pathogenicity features of *M. bovis*, including enhancing colonisation and epithelial cell adhesion (Lysnyansky et al., 2016; Sachse et al., 1996; Sachse et al., 1993). These Vsps also have the ability to induce the expression of both up- and down-regulating cytokines e.g. IFN-γ in leukocytes and

lymphocytes e.g. CD4+ (helper) and CD8+ (cytotoxic) T cells (Bush & Rosenbusch, 2003; Kauf et al., 2007; Rosati et al., 1999). Behrens et al. (1996) demonstrated a Vsp-unrelated immunodominant membrane protein, labelled pMB67, was also involved in *M. bovis* surface antigenic variation.

Antigenic variation is also achieved through chromosomal rearrangement, including deletions, duplications, and insertions. This high-frequency size variation, in specific repetitive blocks, within a locus, may result in a number of size variants of each Vsp (Behrens et al., 1994; Lysnyansky et al., 1996). Variable surface lipoproteins offer *M. bovis* an immense capacity to vary its surface antigens, which presents a challenge to developing effective vaccines and antimicrobials (Maunsell et al., 2011). Further investigation is needed to determine whether these complex mechanisms of antigenic variation are utilized by *M. bovis* to avoid the humoral host responses in natural infections.

2.3.1.2 *Adherence to Host Cell*

Adhesion to the host cell in the target tissue, which is facilitated by cell–surface adhesins of the bacterium, is a prerequisite for colonisation and infection (Razin, 1999; Rottem, 2003). The mycoplasma membrane adhesins are known to have direct contact with the host cell (Sachse et al., 1996), aid mycoplasmal survival and are considered important virulence factors of *M. bovis*. Several adhesins and cytoadherence-related proteins have been identified including P26, a surface-located protein that adheres to embryonic bovine lung (EBL) cells (Sachse et al., 1996); a family of Vsps including VspA, VspB, VspE and VspF (Sachse et al., 2000) and a new Vsp protein (Thomas et al., 2005). *Mycoplasma bovis* NADH oxidase has also been found to behave as an adhesin (Zhao et al., 2017). Other in vitro studies have identified a plasminogen-binding protein, α-enolase (Song et al., 2012), and VpmaX (Zou et al., 2013) as adhesins. In an *in vitro* study investigating adherence rates to various host cell lines, the only non-pathogenic strain studied showed lower adherence rates compared to three CM isolates (Thomas et al., 2003).

Researchers proposed that merging of the host membrane to these adhesins allows an intracellular exchange of components (Razin et al., 1998). Adhesion to the host cell is advantageous for the bacterium, as it can access specific nutrient requirements, including amino acids, lipids and precursors of nucleic acids (Calcutt et al., 2018; Fox, 2012). It is considered that both a fusion of the cell membrane of the mycoplasma bacterium and the host cell, and penetration of mycoplasmal tip organelle into the host cell are potential virulence factors (Razin, 1999).

Contagious mastitis pathogens, unlike non-contagious mastitis pathogens, have the characteristic of being able to adhere to mammary gland epithelial surfaces (Frost et al., 1977). An *in vitro* study showed the ability of *M. bovis* to adhere to a number of differing host cell lines was not correlated to

the pathological background of the isolate, regardless of whether it was from a case of pneumonia, arthritis or mastitis (Thomas et al., 2003). It has not yet been proven if *M. bovis* has the ability to adhere to epithelial cells in the mammary gland (Fox, 2012).

2.3.1.3 *Internalisation into Host Cells*

The ability of *M. bovis* to invade and then survive within the host cell gives it protection against the host's own immune response (Fox, 2012). Mycoplasmaemia has been seen in calves that have been infected with *M. bovis* (D. Adegboye et al., 1995). The pathogen was reported in neutrophils and macrophages, and also in hepatocytes and epithelial cells of bile ducts (Bürki et al., 2015). In other *in vitro* work, *M. bovis* was shown to invade both bovine peripheral blood mononuclear cells (PBMC), including both T and B cells, and erythrocytes (van der Merwe et al., 2010). The internalization in host cells of *M. bovis* could contribute to the dissemination of the bacterium to multiple organ systems by the lymphatic or haematogenous routes. This finding is consistent with the ability of *M. bovis* to spread to multiple body sites of diseased cattle (Biddle et al., 2005; Jain et al., 1969). This internalisation is also considered to afford *M. bovis* protection against antimicrobial therapy (Bürki et al., 2015; Fox, 2012). *Mycoplasma bovis* being able to invade phagocytes aids its survival and allows the pathogen to persist in the animal (Bürki et al., 2015). The mechanism that allows *M. bovis* to survive phagocytosis has not been totally elucidated (Kleinschmidt et al., 2013).

2.3.1.4 *Immunomodulatory Characteristics*

Another virulence factor of *M. bovis* is its' ability to modulate the host immune system to enhance its survival (Fox, 2012). The membrane proteins of *M. bovis*, including both the Vsps and the Vsp-unrelated pMB67, are critical in this modulation as they interact with the host's immune system. While immunomodulatory mechanisms are complex, controversial and not fully understood (van der Merwe et al., 2010; Vanden Bush & Rosenbusch, 2002), there does seem to be consensus that the bacterium can secrete a peptide that inhibits proliferation of host lymphocytes (Vanden Bush & Rosenbusch, 2004). *Mycoplasma bovis* may both stimulate (Razin et al., 1998; van der Merwe et al., 2010) and suppress the immune system of the host (Mulongo, Prysliak, Scruten, et al., 2013). The mechanisms of immune stimulation include the immune response being upregulated by the induction of cytokines, complement being activated, macrophages or T cells (Bush & Rosenbusch, 2003; Jungi et al., 1996; Kauf et al., 2007). Whereas immune suppression takes place by expression of anti-inflammatory cytokines (e.g. IL-10), or suppression of pro-inflammatory cytokines e.g. IFN-γ and TNF-α (Mulongo, Prysliak, Scruten, et al., 2013). Host immune suppression is also achieved by inhibiting lymphocyte proliferation via a lympho-inhibitory peptide (Vanden Bush & Rosenbusch, 2004), suppressing

lymphocyte response to phytohaemagglutinin (Thomas et al., 1990), and the *in vitro* ability of *M. bovis* to induce apoptotic death of bovine lymphocytes (Vanden Bush & Rosenbusch, 2002). However, a delay in apoptosis in *M. bovis*-infected bovine monocytes was noted in another study (Mulongo, Prysliak, Scruten, et al., 2013).

The host immune response to *M. bovis* is hampered by the bacterium binding to neutrophils and inhibiting the oxidative burst (Thomas et al., 1991). Nevertheless, the bacterium can cause immunomodulation of both the cell-mediated and humoral responses (Fox, 2012). Host immune response modulation is consistent with a protracted survival and systemic dissemination of the bacterium in infected cattle (Mulongo, Prysliak, & Perez-Casal, 2013).

2.3.1.5 *Secondary Metabolites and Biofilm Production*

Like other *Mycoplasma* spp., *M. bovis* produces secondary metabolites that are involved in the pathogenesis of *M. bovis* disease (Hames et al., 2009). Secondary metabolites such as H_2O_2 (hydrogen peroxide) and superoxide radicals are known to damage host cells and lead to cell death, lipid peroxidation or ciliary action inhibition (Hames et al., 2009). The enzyme NADH oxidase, an *M. bovis* adhesin, also generates H_2O_2 (Khan et al., 2005). For many *Mycoplasma* spp, including *M. bovis*, H_2O_2 generation is considered as an important virulence factor (Maunsell et al., 2011; Schott et al., 2014).

Host tissue damage sees the recruitment and stimulation of phagocytes, both macrophages and neutrophils, which release lysosomal enzymes, reactive oxygen and nitrogen species (ROS and RNS) (Beckman & Koppenol, 1996; Fligger et al., 1999; Hermeyer et al., 2011). The superoxide anion with nitric oxide or alternatively nitrite with H_2O_2 forms peroxynitrite and subsequently causes nitrative injury (Sugiura & Ichinose, 2011). Mycoplasmal H_2O_2 together with ROS/RNS from macrophages may cause oxidative and nitrative injury, which result in the characteristic caseonecrotic lung lesions seen in cases of *M. bovis* pneumonia (Hermeyer et al., 2011; Schott et al., 2014). Caseonecrotic lesions have recently been reported in *M. bovis* CM (Radaelli et al., 2011) with a similar pathogenesis is believed to result in these lesions suggested.

Some strains of *M. bovis* produce biofilms (McAuliffe et al., 2006). Biofilms are communities of sessile micro-organisms attached to a surface, often surrounded by an extracellular polysaccharide matrix (McAuliffe et al., 2006). Their production contributes to bacterial persistence in both the environment and inside the host, which may lead to disease chronicity (Bürki et al., 2015). Biofilm production also aids survival of the bacteria against both environmental stressors and host defences (Mah & O'Toole, 2001). It has been shown that strains of *M. bovis* that produce biofilms, have better survival in the environment. The biofilms aid in the prevention of desiccation and enable the bacteria to survive in

hotter temperatures, although in one study, the percentage of *M. bovis* bacteria surviving in a biofilm, at 30 hours, was only 0.01% of the initial inoculated dose (McAuliffe et al., 2006). *Mycoplasma bovis* also survives in bedding sand, from infected dairies for at least eight months (Justice-Allen et al., 2010). An increasing appreciation of biofilms in bovine mastitis is emerging (Gomes et al., 2016). While this work was focused on bacterial mastitis pathogens other than *M. bovis*, biofilms were shown to be of importance in pathogenicity. Biofilms maybe biologically important in recurrent infections, antimicrobial response, and in host defence mechanisms.

2.3.1.6 Co-infections with Other Pathogens

Co-infections with other bacteria and viruses play a role in the development of the Bovine Respiratory Disease Complex (BRDC) (Bürki et al., 2015; Maunsell et al., 2011), see 2.4.4 Pneumonia. In an investigation into chronic pneumonia in Canadian feedlot cattle, a synergism between bovine viral diarrhea virus (BVDV), and its ability to cause immunosuppression, and the pneumonia and arthritis caused by *M. bovis* was proposed in the pathogenesis of these mycoplasma syndromes (Gagea et al., 2006; Shahriar et al., 2002). There has not been any specific discussion in the literature on co-infection and potential synergistic effects with other pathogens for *M. bovis* mastitis.

2.3.2 Transmission

While there is a link between transmission (movement of infection from an infected animal to a susceptible or naive animal within an infected population), colonisation (presence of a bacteria on a body or mucosal surface, without causing disease in that animal) and shedding (discharge of an infectious agent into the environment, by excretion, secretion, exhalation or open wounds) in the epidemiology of *M. bovis* mastitis, these will be reviewed separately.

Traditionally *Mycoplasma* spp. including *M. bovis,* have been considered highly contagious pathogens (González & Wilson, 2003) known to colonise mucosal surfaces in cattle, including the nose, eyes, ears, mammary gland, respiratory tract, prepuce, vagina and tonsils (Fox et al., 2005; Maunsell et al., 2012). *Mycoplasma* spp. are transmitted via the secretions from these mucosal surfaces.

The literature has focussed on the premise that *M. bovis* mastitis, while contagious in nature, is mostly transmitted during the milking routine, from the udder, which is the reservoir for infection. This transmission may be via fomites, milkers' hands and intramammary syringes of antimicrobial preparations. As opposed to environmental mastitis, where the primary reservoir for the pathogen (e.g. *Streptococcus uberis*), is the environment and not the infected udder (Smith et al., 1985), stringent hygiene practices at milking have played a major role in controlling the more traditional

contagious mastitis bacteria (e.g. *Staphylococcus aureus* and *Streptococcus agalactiae)*. While indirect transmission of *M. bovis* from udder to udder during the milking routine is recognised as a major route of transmission (Fox, 2012), researchers found that despite high hygiene standards, post milking teat disinfectants, attention to detail with milking routine and shed maintenance, the prevalence of *M. bovis* mastitis on infected farms still increased (Enger et al., 2015). Similarly Punyapornwithaya et al. (2012) showed it was not possible to associate elimination of mycoplasmas from a dairy herd with any milk hygiene or control practices.

The transmission of *Mycoplasma* spp. in dairy cows on an infected dairy platform, where CM is present may occur via two mechanisms (Fox, 2012). Firstly, via large droplets and short-range aerosols due to the presence of *M. bovis* in respiratory secretions and milk. This is direct transmission or transmission via nose-to-nose contact, where the bacteria may be shed through an external mucosal surface of an infected or colonised animal to a naive animal (Calcutt et al., 2018; Maunsell et al., 2011). Secondly, indirect transmission via milking equipment and other fomites (Fox, 2012), or feeding of infected colostrum/milk to calves (Maunsell & Donovan, 2009). Dissemination of *M. bovis* within an infected animal can also occur where *M. bovis* spreads haematogenously from an infected organ, body system or mucosal surface, to the mammary gland or in reverse, with initial infection of the udder followed by spread to other systems or mucosal surfaces. Isolates from the respiratory and urogenital systems, as well as the mucosal surfaces of the ear and eye, have been of the same strain type as isolates found in the mammary gland (Biddle et al., 2005). In an infected herd, multiple potential transmission routes could play a role in the transmission of *M. bovis.*

Other potential transmission routes are noted in the literature. Vertical transmission of *M. bovis* mastitis has been suggested following a case of mastitis in pre-pubertal heifers, where these heifers were infected with the same strain as their infected dams and other herd mates (Fox et al., 2008). *Mycoplasma* spp. can survive in the environment for some months at varying temperatures and on different materials (Justice-Allen et al., 2010; Wilson et al., 2011). However, further research is necessary to define the role of the environment as a reservoir for Mycoplasma mastitis (Fox, 2012).

Seminal vesiculitis in bulls has been experimentally induced in *M. bovis* studies (Ruhnke, 1994) and *Mycoplasma* spp. have been isolated from the semen of bulls (Ungureanu et al., 1986). Recent outbreaks of *M. bovis* mastitis in two naive Finnish dairy herds where the introduction of *M. bovi*s infection was via semen used for artificial insemination (AI) is strongly suspected (Haapala et al., 2018). The practice of feeding colostrum or contaminated milk from cows with *M. bovis* mastitis has been linked to calves becoming infected (Maunsell et al., 2012). The *M. bovis* nasal prevalence and colonisation in calves fed infected colostrum or contaminated milk is higher than in calves fed clean milk (Bennett & Jasper, 1977b).

2.3.3 Colonisation

The ability of *M. bovis* to colonise numerous mucosal surfaces has been well documented (Fox et al., 2005; Hazelton et al., 2018; Punyapornwithaya et al., 2010). Similarly, haematogenous spread of *M. bovis* from a foci of *M. bovis* arthritis and/or bronchopneumonia to allow mammary gland colonisation, potentially leading to *M. bovis* CM, has been postulated (Pfützner & Sachse, 1996; Punyapornwithaya et al., 2010).

Early work suggested that the upper respiratory tract (URT) is the initial colonisation site for naturally infected calves (Bennett & Jasper, 1977b; Brys & Pfützner, 1989). In an experimental study, the colonisation of the URT was demonstrated by oral inoculation with *M. bovis* in calves which were slaughtered 14 days later. The tonsils, both palatine and pharyngeal, had high microbial loading at slaughter, while the nasal passages (deep nasal swabs) did not. Otitis media was also clinically diagnosed in these calves. Recent NZ work (Buckle et al., 2020), further supports the colonisation of palatine tonsils in naturally infected calves, where from 51 palatine tonsils that were swabbed at slaughter, 92.7% (95% CI 82.4-98.0%) were qPCR positive for *M. bovis*, compared to 12.7% (95% CI 5.3-24.5%) for swabs from mainstem bronchi. These calves originated from a *M. bovis*-infected herd.

The mucosal surfaces of the nasal cavities, eyes, ears, mammary gland, respiratory tract, vagina, prepuce, and tonsils are known colonisation sites for *M. bovis* (Biddle et al., 2005; Hazelton et al., 2018; Maunsell et al., 2012). The easily accessible sites, namely the nasal cavity, which is sampled routinely in young animals under eight months of age (Bennett & Jasper, 1977b), eyes, ear, milk and ear (Fox et al., 2005; Maunsell et al., 2012), are usually used in experimental or clinical diagnosis where isolation of the bacteria is sought to confirm the colonisation and/or infection status of the cow/calf or herd. Deep nasopharyngeal swabs have been used for investigation into respiratory disease of mixed aetiology in calves (Godinho et al., 2007). A colonised or infected animal may be an asymptomatic subclinical carrier or clinical case. To aid in *M. bovis* diagnosis, or support control of *M. bovis* in asymptomatic cows, palatine tonsil swabs can also be taken from live cattle in the field by a clinician. There is no published literature on the colonisation of the palatine tonsil in the *M. bovis* CM cow or clinically asymptomatic cow in an infected dairy herd.

Two important studies have further elucidated our understanding of *M. bovis* colonisation. Their study objectives, study populations and testing methodologies were slightly different. Firstly, an Idaho study (Punyapornwithaya et al., 2010) in a herd that had experienced *M. bovis* mastitis and was evaluating the association between *M. bovis* mastitis and colonisation at different body sites in asymptomatic carriers. The ears, eyes, nose, vulvovaginal tract, and milk of asymptomatic carriers were swabbed for

culture four times over a year. *Mycoplasma* spp. isolated were speciated and fingerprinted, using PFGE. This work showed the nasal mucosa was the most likely site to be colonised with *M. bovis* cultured from 21% (18/84) cows, 3.6 % (3/84) of cows' eyes were culture positive, and no isolations from ears. If *M. bovis* was isolated from a cow at one site at one time point, sampling of this site was never repeated during subsequent sampling, nor did *M. bovis* isolation from a body site preceded mastitis. Also, in the initial stages of this outbreak of *M. bovis* mastitis colonisation of different body sites with the outbreak strain was common, but the prevalence of colonisation decreased over time.

Secondly, Australian work Hazelton et al. (2018) swabbed the accessible mucosal surfaces of the eye, nose and vagina of 16 cows that developed *M. bovis* CM one to two weeks prior to sample collection. The *M. bovis* CM had been diagnosed by milk qPCR and 15/16 cows were serum ELISA positive on serum (using Bio-X kit with an optical density coefficient (ODC%) cut point of 37%, and diagnostic sensitivity of 93.8%). From the three mucosal sites, *M. bovis* was only detected in three (18.8%) vaginal swabs, with no detection from ear or nose swabs (Hazelton et al., 2018). This low prevalence of *M. bovis* on mucosal surfaces suggests that colonisation of these sites may be sporadic, given the analytical sensitivity of *M. bovis* qPCR is high.

2.3.4 Shedding

The shedding of *M. bovis* from mucosal surfaces of infected cows is known to be intermittent and inconsistent (Biddle et al., 2003; Hazelton et al., 2018; Wilson et al., 2009). This intermittent shedding is commonly described with chronic and subclinical mastitis (SCM) cases (Bushnell, 1984; Gonzalez et al., 1992; Jasper, 1981a). The reasons for this intermittent shedding are not fully understood, but hypothesized reasons are the stress status of the animal and the time course of the infection (Calcutt et al., 2018).

Shedding of *M. bovis* from infected animals may last from a few weeks to several months (Punyapornwithaya et al., 2010) or possibly years (Bayoumi et al., 1988). Prolific shedding (>10^6 cfu/ml by milk culture) of *Mycoplasma* spp. is often seen in cases of mycoplasma CM (Biddle et al., 2003). The limit of detection for *M. bovis* in milk culture is reported as 10^2 cfu/ml (Cai et al., 2005) to 10^3 cfu/ml (Parker et al., 2018) while 10^2 cfu/ml is the accepted PCR limit for detection of *M. bovis* in milk samples (Clothier et al., 2010; Rossetti et al., 2010). An American study that followed 10 infected cows for a 28-day period, assessing the frequency of shedding with chronic mycoplasma intramammary infection (IMI), found variable shedding (Biddle et al., 2003), where *Mycoplasma* spp. were isolated, by culture, from 71% of composite milk samples (CMS). At a cow level, 10% of the time cows shed between 10^2 and 10^4 cfu/ml, 1% of the time between 10^4 and 10^5 cfu/ml, and 60% of the time >10^5 cfu/ml. In another study, a cow with chronic mastitis from *M. bovis* infection was reported to not shed for a 56-

day period (González, 1999). These findings in part explain why intermittent shedding and asymptomatic carriage can hinder consistent detection of *M. bovis*, especially at an animal level (Hazelton et al., 2018), which increases the risk of a misdiagnosis (Biddle et al., 2003). Also, asymptomatic infected carrier cows are able to shed mycoplasma bacteria in milk or nasal secretions for months to years with no clinical signs (Calcutt et al., 2018; Nicholas et al., 2008). More research is needed into the shedding of *M. bovis* in mastitis.

2.3.5 Prevalence

There are many *Mycoplasma* spp. and *M. bovis* prevalence studies in the literature. The 2018 National Mastitis Council Research Committee Report (Lopez-Benavides et al., 2018) reported that while IMI and mastitis denote different entities, these terms have been used incorrectly interchangeably over many years. Three states of *M. bovis* infection can be considered. Firstly, *M. bovis* seropositivity in milk or blood, with *M. bovis* not detected in the milk, by culture or qPCR, does not reflect an IMI or mastitis but rather a systemic response to previous *M. bovis* infection. Secondly, *M. bovis* detected in milk but no mammary gland change indicative of mastitis is a *M. bovis* subclinical mastitis (SCM), and an IMI present. Thirdly, *M. bovis* detected in milk and mammary gland changes indicative of mastitis is a *M. bovis* CM, and an IMI present. Studies report differences in the herd-level prevalence of *M. bovis* infection and within-herd prevalence of *M. bovis* infection. Together with *M. bovis* subclinical IMI from a small number of studies, they are presented in Table 1. (Different methodologies have been used, and these have been reported under Diagnostic test used).

Herd-level Prevalence of M. bovis

Estimated *M. bovis* herd-level prevalence varied between countries, from 0.9% in Australia to 5.4% in Greece. Most studies involved sample collection from a region (i.e. state or province) as opposed to sample collection from across the country. While all estimates are derived from testing of BTM samples, different studies used *M. bovis* PCR, culture, or ELISA. Some studies reported the herd-level prevalence of *Mycoplasma* spp. (Fox, 2012). There is limited herd-level seroprevalence work published, though a Swiss study (Burnens et al., 1999) found that 47% dairy herds in the canton of Jura, Switzerland were BTM *M. bovis* ELISA positive.

Mycoplasma bovis Subclinical Mastitis

The study of *M. bovis* SCM using culture methodology has historically been considered both costly and challenging (Fox, 2012). More recently qPCR technology has allowed a more extensive investigation of SCM. The literature offers a small dataset of studies to evaluate *M. bovis* SCM. Two Estonian studies (Timonen et al., 2017; Timonen et al., 2020) that looked at *M. bovis* IMIs presented very different

results. One study of a 600-cow *M. bovis*-infected herd had a within-herd prevalence of *M. bovis* IMI, using milk qPCR, of 17.2% (Timonen et al., 2017). A more recent repeated cross-sectional study of four herds with endemic *M. bovis* infection, where all cows were milk qPCR tested three times over a six-month period, had a much lower prevalence (Timonen et al., 2020). The two studies were not compared, and no explanation given as to the marked difference. Another recent Australian study (Hazelton et al., 2020) demonstrated a very low (0.0 to 0.2%) apparent prevalence (AP) herd-level of *M. bovis* IMIs, across a study in four herds with clinical *M. bovis* cases. The samples were collected after CM cases were removed from the herds. More work on the prevalence of *M. bovis* IMI is needed to fully explain and quantify this problem. These studies suggest that while most farms have a low prevalence of *M. bovis* SCM, occasional farms may have a high prevalence.

Mycoplasma bovis Clinical Mastitis

Estimation of the cow-level prevalence of *M. bovis* CM has been more difficult (Fox, 2012), with several studies presenting prevalences only at a *Mycoplasma* spp. level (Bradley et al., 2007; Hertl et al., 2011). Nevertheless, in North America, *M. bovis* is considered one of the most economically important mastitis pathogens (Rosengarten & Citti, 1999). In a US study, Brown (Brown et al., 1990) reported that, in herds with *M. bovis* up to 70% of mastitis cases, were infected with *M. bovis*. In contrast, two multi-herd studies reported low prevalences of *M. bovis* CM. Firstly, 19 known *M. bovis* infected dairy farms in Finland, with a median size of only 61 cows, were followed over a two-year period. Vähänikkilä et al. (2019) reported only a few cases of *M. bovis* CM. Fifty-one cases were recorded over the 19 farms, with eight farms only experiencing one case of *M. bovis* CM, with the range of cases/farm being one to eight. Milk qPCR was used on all mastitis cases. Of note, 88% of new clinical cases were reported within eight weeks of the index case. Secondly, 19 case herds were also enrolled in a Swiss study, on farms with confirmed *M. bovis* infection. Two visits to each farm were made to milk sample CM cows, including cows with multiple quarter CM. Milk qPCR positive cows were only found at the first visit and accounted for 18/742 CM cases (2.4%; 95%-CI: 1.5-3.8%).

A large Saudi Arabian study (Al-Abdullah & Fadl, 2006) showed the prevalence of cases of *M. bovis* CM was highest in heifers in their first lactation, post calving. This finding agreed with early American work (Bayoumi et al., 1988). Contrary to these studies, other American work notes that despite using teat disinfectants post milking as part of the milking routine, *M. bovis* CM prevalence increased with cow age (Fox, 2012; Zadoks, 2015).

Table 2-1 Prevalence of Mycoplasma bovis

(i) Herd-Level Prevalence

Reference (or study)	Diagnostic test	Apparent Prevalence	Notes
Australia (2014) [1]	BTM PCR	0.4%	Estimated true prevalence = 0.9% (95% CI 0.1-3.7%); diagnostic sensitivity 76% (95% CI 34-98%)
Australia (2020) [2]	BTM culture	2.4%	*M. bovis*
Belgium (2012) [3]	BTM culture	1.5%	*M. bovis*
Greece (2007) [4]	BTM culture	5.4%	*M. bovis*
Norway (2015) [5]	BTM ELISA	4% *	*M. bovis* * ODC 37% cut off for North Norway
Israel (2016) [6]	BTM culture & individual culture	<1-3%	*M. bovis* (sample 2009-2014)
Japan (2019) [7]	BTM PCR	3.8%	*M. bovis* (95% CI 2.6%, 5.4%)
USA (2012) [8]	BTM culture	<3%	Mycoplasma spp. North East/Midwest States
		9.4%	Mycoplasma spp. West (large herds)
Mexico (2008) [9]	BTM culture	55%	Mycoplasma spp.

(ii) Within-Herd Prevalence

Reference (or study)	Diagnostic test	Apparent Prevalence	Notes
Ghazaei (2006) [10]	Cow culture	100%	Mycoplasma spp.

(iii) *M. bovis* Within-Herd Intramammary Infections (IMI) (subclinical infection)

Reference (or study)	Diagnostic test	Prevalence
USA (2014) [11]	Individual milk culture & qPCR	Herd 1: 2.8% (n=1210, 95% CI = 1.9; 3.7) Herd 2: 0.6% (95% CI = 0.0; 1.4)
Estonia (2016) [12]	Individual milk qPCR	17.2% (n=611)

Estonia (2020) [13] — Individual milk qPCR

	Cow #	Herd Test 1	Herd Test 2	Herd Test 3
Herd 1	591	4.7%	1.0%	0.8%
Herd 2	1633	3.4%	0.4%	0.4%
Herd 3	522	12.3%	4%	3.2%
Herd 4	1035	7.8%	2.8%	4.9%

Australia (2020) [14] — Individual milk qPCR

	Cow #	Seroprevalence	Main Herd IMI qPCR
Herd 1	447	76	0.2%
Herd 2	475	40	0.0%
Herd 3	816	20	0.1%

References

[1] (Morton et al., 2014) [2] (Hazelton et al., 2020) [3] (Passchyn et al., 2012)

[4] (Filioussis et al., 2007) [5] (Nielsen et al., 2015) [6] (Lysnyansky et al., 2016)

[7] (Murai & Higuchi, 2019) [8] (Fox, 2012) [9] (Miranda-Morales et al., 2008)

[10] (Ghazaei, 2006) [11] (Murai et al., 2014) [12] (Timonen et al., 2017)

[13] (Timonen et al., 2020) [14] (Hazelton et al., 2020)

2.3.6 Incidence

Incident rates of *M. bovis* CM are not widely discussed in the literature. Swiss work (Aebi et al., 2015) reported the incidence rate of *M. bovis* CM of 0 – 0.1 case per animal year at risk, and clinical pneumonia at 0.1 – 0.6 cases per animal year at risk. Using different indices, American work saw *M. bovis* CM incidence rates in the milking herd of 0.01 cases per 100 cow-days at risk, and 1.7 cases per 100 cow-days at risk in the hospital pens (Punyapornwithaya et al., 2011).

2.3.7 Herd-Level Risk Factors

There are many risk factors for outbreaks of mycoplasmal disease on a dairy farm, especially outbreaks of CM, including increased herd size, purchase and introduction of stock, the seasonal movement of different classes of animals, stress factors and also infected semen used in AI (Aebi et al., 2015; Bayoumi et al., 1988; Murai & Higuchi, 2019; Nicholas et al., 2016).

Early work by Thomas et al. (1981) showed there was a positive and significant correlation in herds that had mycoplasma mastitis between herd size and culling percentage. From a large two-year study of 650 herds in the USA, large herd size was shown to be significantly correlated with a BTM culture of *M. bovis*, where herd size was measured by total milk production i.e. milk production was used as a proxy for cow numbers (Fox et al., 2003). Conclusive evidence for herd size as a risk factor was demonstrated when BTM prevalence of *Mycoplasma* spp. increased with herd size from 2.1% for herds <100 cows, 3.9% for 100-499 cows, and 21.7% for herds >500 cows (McCluskey et al., 2003). A marked increase in the incidence of mycoplasma mastitis cases was seen in Israel with the expansion of herd size to >600 cows (Lysnyansky et al., 2016). While management practices in large herds may lead to high cow turnover, large numbers of stock movements and purchases increase the risk of introducing an infected animal into a herd (Fox et al., 2003; Pinho et al., 2013). Therefore, it is deemed the large herd size itself, irrespective of management practices, affords greater opportunity for introduction of mycoplasmas and enables infection to be more easily maintained in the herd (Nicholas et al., 2016).

Stock introduction from an outside source is the most significant risk factor for mycoplasmal mastitis, including *M. bovis* (Punyapornwithaya et al., 2010). This introduction may include infected asymptomatic carriers moving into a naive herd or the movement of stock for grazing or shows where there is the potential for mixing of uninfected and infected cattle. In one Swiss study (Burnens et al., 1999) where multivariate analysis was used to identify possible risk factors, animal purchase was the only variable significantly associated with herd serological status. Another retrospective case study, which used logistic regression, identified movement of animals, high mean herd milk production, one

brand of milking cups, and other stress factors as herd-level risk factors (Aebi et al., 2015). In a Japanese study, after controlling for herd size, purchase of cows and corporate farming models where frequent movement of stock was common, were risk factors. (Murai & Higuchi, 2019).

Asymptomatic carriers may carry *Mycoplasma* spp. until a stress factor precipitates clinical disease (Fox, 2012). Calving is deemed a common stress factor (Bushnell, 1984; Punyapornwithaya et al., 2010). High stocking density, mouldy feed, high in-barn temperature, and concomitant disease are also noted risk factors (Aebi et al., 2015). Environmental, physical or nutritional stress factors may cause host immunosuppression that precipitates clinical outbreaks (Bayoumi et al., 1988; Boothby et al., 1986; Jasper, 1981a).

In an univariable logistic regression model looking at the herd-level presence of *M. bovis* and associated risk factors, peri-calving conditions such as metritis, hypocalcaemia, clinical ketosis, and abomasal displacements were noted as concomitant diseases (Aebi et al., 2015). Mouldy feed where the fusarium toxin, deoxynivalenol, is present is another possible risk factor. This toxin is able to decrease the immune response, by reducing neutrophil phagocytic function (Fink-Gremmels, 2008). High producing herds are at higher risk of being in a negative energy balance (NEB), especially if poorly fed around calving, and would therefore be more predisposed to infectious disease (Goff, 2006). Two studies (Aebi et al., 2015; Feenstra et al., 1991) demonstrated that *M. bovis* case herds had higher mean milk production compared to control herds.

There are also other risk factors. The dry cow period is a risk period for *M. bovis* mastitis outbreaks (Bicknell et al., 1983; Otter et al., 2015). The exact mechanism of dry cow mastitis needs further investigation (Nicholas et al., 2016). Production systems where potentially infected waste milk or colostrum is fed to calves increases transmission risk. Pasteurisation of milk being fed to calves is recommended (Foster et al., 2009). The mixing of infected calves with cows and vice versa is a risk factor as direct contact, large droplets, and aerosols are known to spread respiratory disease (Lysnyansky et al., 2016).

While seminal vesiculitis in bulls has been experimentally induced in *M. bovis* studies (Ruhnke, 1994), there has been isolation of *Mycoplasma* spp. from the semen of bulls (Ungureanu et al., 1986). Artificial insemination (AI) of cows with *M. bovis* infected semen is considered a route of infection (Pfutzner, 1990; Wrathall et al., 2007) and hence another risk factor. A recent Finnish investigation (Haapala et al., 2018) found that *M. bovis*-infected semen used for AI in two self-replacing herds was the likely source of infection that caused *M. bovis* CM outbreaks. *Mycoplasma bovis* can persist in frozen semen for years (Pfutzner, 1990). Embryo transfer (ET) is also a potential risk factor (Bielanski et al., 2000).

2.4 Clinical Manifestations of *Mycoplasma bovis*

There are numerous clinical manifestations associated with *M. bovis* infection. Mastitis, arthritis, tenosynovitis, pneumonia, and reproductive syndromes are seen in adult cattle, including dairy, beef and feedlot cattle (Maunsell et al., 2011; Pfützner & Sachse, 1996). While the most common clinical syndromes observed in young calves aged two to six weeks old are pneumonia (Nicholas et al., 2002), arthritis (Maunsell & Donovan, 2009), and otitis media (Maunsell et al., 2012), less common presentations include tenosynovitis (Adegboye et al., 1996), decubital abscesses, meningitis (Stipkovits et al., 1993), and keratoconjunctivitis (Levisohn et al., 2004). For both experimental and natural infections that are *M. bovis*-associated, there is variability in disease expression. Epidemiologically, the maintenance and dissemination of *M. bovis* in populations of cattle does not seem to rely on clinical disease (Maunsell et al., 2011).

2.4.1 Mastitis

2.4.1.1 Clinical Mastitis

Mycoplasma bovis is considered to be a contagious mastitis pathogen (Royster & Wagner, 2015; USDA, 2008). *Mycoplasma bovis* mastitis at the herd level can vary from endemic subclinical IMI, to mild CM, to outbreaks of severe clinical disease. Chronic infections can also occur (Maunsell et al., 2011; Timonen et al., 2020). Mastitis outbreaks may be diagnosed concurrently with arthritis (Wentink et al., 1987; Wilson et al., 2007) and/or pneumonia caused by *M. bovis* (Petersen et al., 2018). Outbreaks of CM vary in time to resolution. Outbreaks have varied in duration, from two months, to a year, to several years (Bayoumi et al., 1988; Fox et al., 2003; Jasper, 1981b; Punyapornwithaya et al., 2012). In contrast, in some herds with clinical disease attributable to *M. bovis* infection resolves after a period of time, in the absence of intervention (Nicholas et al., 2016). *Mycoplasma* spp. are known to cause mastitis at any stage of lactation, including in dry cows and pre-pubertal heifers (Bicknell et al., 1978; Fox et al., 2008; Maunsell et al., 2011). While there are many reported case studies of *M. bovis* mastitis outbreaks where <5 % of cows develop CM (Aebi et al., 2015; Al-Abdullah & Fadl, 2006; Vähänikkilä et al., 2019), Pfützner and Sachse (1996) claimed that in an outbreak of *M. bovis* mastitis, usually >20% of cows are affected. While not stated, it would be assumed the reference was to CM.

Outbreaks of clinical disease, including mastitis, are often diagnosed in the postpartum period and early to peak lactation when cows are under the most stress (Bayoumi et al., 1988; Pfützner & Sachse, 1996; Radaelli et al., 2011). In a multi-farm Finnish study most of the CM was diagnosed within eight weeks of the index case (Vähänikkilä et al., 2019). Early work reported the incubation period (IP), defined as the period from exposure to infection through to the first appearance of clinical signs, of

M. bovis CM to be two to six days and dose dependent (Pfützner & Sachse, 1996). This short IP has been challenged by other work that has suggested the IP is 13.6 days (Punyapornwithaya et al., 2011). While several factors were considered to potentially affect the IP, including herd management, stress on the cows, and any co-infection, further studies are needed to determine the minimum and maximum IP for *M. bovis* CM (Calcutt et al., 2018). The diagnosis of latent *M. bovis* infection is challenging (Calcutt et al., 2018).

Early experimental work with *M. bovis* CM recorded the spread of mastitis to all four quarters (Jain et al., 1969). However, four quarter mastitis is not always present in cases of *M. bovis* CM, where cows may develop mastitis in one, two, three or all four quarters (Byrne et al., 1998; Jasper, 1981a; Maunsell et al., 2011; Pfützner & Sachse, 1996). A pattern of the second quarter affected, being on the same side as the first affected quarter is also described, the right side of the udder being more common (Byrne et al., 1998; Jasper & Al Aubaidi, 1974). Most case studies on outbreaks of *M. bovis* mastitis do not describe the mastitis to a quarter level, rather summarised as the mastitis as affected quarters, multiple quarters or plainly the number of cows with mastitis (Al-Abdullah & Fadl, 2006; Pothmann et al., 2015; Radaelli et al., 2011). There is broad agreement that treating clinical cases of *M. bovis* CM with antibiotics has limited effect (Ayling et al., 2014; Calcutt et al., 2018).

A common characteristic of *M. bovis* CM is that the infected cows are generally healthy and signs of being systemically affected are mild (Al-Abdullah & Fadl, 2006; Jain et al., 1969; Nicholas et al., 2016; Pfützner & Sachse, 1996). Both experimentally (Jain et al., 1969) and naturally infected cows may show initial transient udder swelling, which has been described both as painful (Wentink et al., 1987) or not painful (Radaelli et al., 2011). A rapid decline in milk production from infected quarters is a common finding (Al-Abdullah & Fadl, 2006; Jain et al., 1969; Pfützner & Sachse, 1996). Another important observation in *M. bovis* CM is that infected quarters may become agalactic (Jain et al., 1969; Pfützner & Sachse, 1996). Enlargement of the supramammary lymph nodes has also been documented (Al-Abdullah & Fadl, 2006). A common characteristic for *M. bovis* CM is a failure to recover and cows are consequently culled (Nicholas et al., 2016; Pfützner & Sachse, 1996).

The progression of changes in the gross appearance of milk secreted by cows with experimental *M. bovis* mastitis has been described. Initially, milk was watery with occasional clots or flakes that settled to a fine sediment in a watery supernatant. This initial appearance progressed to a serous yellow fluid and to a thick purulent to caseous secretion over two to three weeks (Jain et al., 1969). The descriptions of milk from naturally exposed cows vary but include milk 'containing sandy sediment, with brown colouring and rice like structure' (Tolboom et al., 2008), odourless but varied from a milk that was brown in colour to almost a purulent discharge and yellow and flaky secretions (Pothmann et al., 2015). In more chronic cases, the milk separated into a sediment and a supernatant-like serum

(Byrne et al., 1998). There was agreement that the gross appearance of milk was non-specific and variable.

Numerous outbreaks of *M. bovis* CM have been reported internationally. There are reports from small housed European herds to large 5000 cow herds in the Middle East, which are housed due to extreme weather conditions (Al-Abdullah & Fadl, 2006). A large Australian study which considered *M. bovis* SCM infections across four herds in Victoria and Tasmania, where up to 45 CM cows in a herd had been culled prior to the start of the study, gave no data about the outbreaks (Hazelton et al., 2020). There is a paucity of published literature on *M. bovis* CM in large (> 500 cows), pasture-based, seasonal-calving farms, where the epidemiology may be different to other reported outbreaks.

2.4.1.2 *Somatic Cell Count*

The use of the somatic cell count (SCC) as an indicator of mastitis in dairy cows is common (Ghadersohi et al., 1999). While the reporting of individual somatic cell counts (ISCCs) and bulk milk somatic cell counts (BMSCCs) in cases of *M. bovis* mastitis, both clinical and subclinical IMIs, offer inconsistent findings, the cut point thresholds may vary and care is needed in interpretation. Biddle et al. (2003), in a study looking at subclinical mycoplasma IMIs, including *M. bovis*, where ISCCs were generally high, found a linear correlation between the log of cfu/ml in CMSs and QMSs and the log of ISCCs. This finding suggests cows with higher ISCCs were shedding higher numbers of organisms. However, some low-ISCC cows were also found to be shedding mycoplasmas. Biddle et al. (2003)'s conclusion was that mycoplasmal IMI could not be ruled out based on a low ISCC, in agreement with Ghadersohi et al. (1999) who also reported cows with low or normal ISCCs shedding *M. bovis.*. An Australian study (Hazelton et al., 2020) where an infected herd was split in three based on ISCC (<100,000 cells/ml to form a low-risk herd, 100-200,000 cells/ml a medium-risk herd, >200,000 cells/ml a high-risk herd), agreed with this conclusion. A BTM PCR positive result for *M. bovis* for all three herds after the split suggests it is not possible to identify cows infected with *M. bovis* using ISCC alone.

A Japanese investigation (Higuchi et al., 2013), offered a more clear-cut outcome. Using Schukken's threshold (Schukken et al., 2003) of >200,000 cells/ml milk to distinguish between cows with and without mastitis, they found, by culture, 93% of 124 *M. bovis* CM quarters had an ISCC >200,000 cells/ml and the other 7%, still *M. bovis* culture positive, had ISSCs <200,000 cells/ml and had no clinical signs of mastitis (i.e. had subclinical IMI). In an Italian outbreak of *M. bovis* CM, Radaelli et al. (2011) reported lower milk quality among cows with CM, with most clinical cows having an ISCC > 1,000,000 cells/ml.

While the BMSCC fluctuated and was actually lower the summer of a *M. bovis* clinical outbreak (Wilson et al., 2007) than the mean BMSCC for the preceding year, in a small Austrian herd, the BMSCC rose from 130,000 to 352,000 cells/ml during an outbreak (Pothmann et al., 2015).

2.4.1.3 Other Pathogens in M. bovis Outbreaks

Limited published information is available on the presence of other bacterial pathogens during *M. bovis* CM outbreaks. A large 664-herd American study (Fox et al., 2003) that ran over more than two years used BTM culture to test for *Mycoplasma* spp., *Staphylococcus aureus (S. aureus)* and *Streptococcus agalactiae (S. agalactiae).* Their conclusion was the presence of *Mycoplasma* spp. in the BTM was not related to other contagious mastitis pathogens being present, suggesting there was a difference between the aetiology and transmission *of S. aureus* and *S. agalactiae* and mycoplasma mastitis.

From a large 522-cow Estonian herd study, which had experienced *M. bovis* CM three years prior to this study (Timonen et al., 2017), that was looking at within-herd prevalence of *M. bovis* IMI, individual cow composite milk samples (CMS) were processed through a Multiplex qPCR. Mastitis pathogens were detected in 200 samples, with *M. bovis* the sole pathogen detected in 15% of these; *M. bovis* and *S. agalactiae* in 19.5%*; M. bovis, S. aureus and S. agalactiae* in 7.5% and *M. bovis and S. aureus* in 3%. This large data set in a subclinical IMI study contrasts with data from *M. bovis* CM outbreaks, where data are limited. In a 14-cow herd Austrian outbreak (Pothmann et al., 2015), 10/14 cows were culture positive for *M. bovis* on CMS, and from these cows on quarter milk samples (QMS), two cows had a single quarter *S. uberis* growth; two cows had non-aureus staphylococci in either one or two quarters; and a final cow had Strep D (*S. dysgalacatiae*) in a single quarter. As *M. bovis* was diagnosed on CMS, QMS growths cannot be matched with the presence of *M. bovis* at a quarter level. From an Italian outbreak of *M. bovis* CM where 45/122 cows had CM, 32 sampled cows were culture positive for *M. bovis* on CMS, with no other pathogens isolated from these samples. *Mycoplasma bovis* was not cultured from 75 cows in this herd which were not showing signs of mastitis, while IHC of the mammary tissues of two of the clinically affected cows showed considerable intralesional *M. bovis* antigen but no other bacteria or fungi were found (Radaelli et al., 2011).

2.4.2 Otitis Media in Calves

Calves that were experimentally infected with *M. bovis* by the oral route showed clinical signs of otitis media within 7-9 days post inoculation (Maunsell et al., 2012), with a range of clinical signs, including pyrexia, decreased appetite, epiphora, and head and ear shaking. Calves often exhibited signs of deficits in cranial nerve VII (facial nerve) function, which manifested clinically as a unilateral or bilateral

ear droop (Brown et al., 1998; Walz et al., 1997) and/or blepharoptosis (Maunsell & Donovan, 2009). Calves with otitis media were also likely to present with respiratory disease or arthritis (Lamm et al., 2004; Maunsell et al., 2012).

Maunsell et al. (2012) proposed colonisation of the pharyngeal tonsils by *M. bovis* occurred after the ingestion of *M. bovis*-infected milk, and the from the tonsil, *M. bovis* infection can be disseminated via the eustachian tube to the middle ear, and the tympanic bullae. Calves with otitis media also have a purulent ear discharge, which is associated with tympanic membrane rupture (Walz et al., 1997). Otitis interna is a reported sequelae of otitis media, and some calves may also develop meningitis as a complication of otitis interna (Van Biervliet et al., 2004).

2.4.3 Genital Disorders

Experimentally induced *M. bovis* disease has been associated with a small number of abortions and genital infections in cows (Pfützner & Sachse, 1996; Stalheim & Proctor, 1976) and seminal vesiculitis in bulls (LaFaunce & McEntee, 1982). Other experimental work in cows reported oophoritis, salpingitis, endometritis and abortion (Ruhnke, 1994). There is limited detail in the literature describing abortion post natural exposure to *M. bovis* (Hermeyer et al., 2012; Stipkovits et al., 1993). British work (Houlihan et al., 2007) reported three abortions at the peak of a *M. bovis* mastitis and arthritis outbreak in a small herd of 120 cows, where *M. bovis* was cultured from the viscera on one aborted calf. Other common causes of abortion were not detected. Systemic mycoplasmosis from *M. bovis* infection resulting in dystocia and abortion in a North American bison herd has been reported (Register et al., 2013).

Further experimental work proved *M. bovis* could be shed in the semen of a bull with a genital tract infection. Researchers proposed that another route of transmission would be the use of infected semen (Pfutzner, 1990). The literature contains little supporting evidence that natural infection with *M. bovis* plays a major role in reproductive losses (Maunsell et al., 2011). However, recent Finnish work (Haapala et al., 2018) has described two outbreaks of *M. bovis* CM where introduction is suspected to have been via the use of semen, for AI, containing *M. bovis*.

2.4.4 Pneumonia

Cattle of any age can develop *M. bovis* pneumonia, including calves, mature dairy cows and feedlot cattle (Maunsell et al., 2011). Animals with *M. bovis* pneumonia may also develop otitis media and/or arthritis (Adegboye et al., 1996; Gagea et al., 2006). Clinically cases of pneumonia due to *M. bovis* infection are indistinguishable from cases of pneumonia caused by other pathogen(s). Presenting signs

are considered non-specific and may include pyrexia, dyspnoea, high respiratory rate, nasal discharge and poor appetite (Adegboye et al., 1996; Maunsell & Donovan, 2009). Abnormal breathing sounds, similar to other pneumonias, may be heard on auscultation of the chest. Emaciation is a sequelae of chronic pneumonia in calves (Ames, 1997).

In natural respiratory tract infections *M. bovis* is commonly associated with infection with other pathogens, including both bacteria (e.g. *Pasteurella multocida, Mannheimia haemolytica* and *Histophilus somni*) and viruses (e.g. bovine parainfluenza virus 3 (BPI3), bovine respiratory syncytial virus (BRSV), and bovine herpes virus 1 (BHV-1)). Together these pathogens play a role in Bovine Respiratory Disease Complex (BRDC) (Bürki et al., 2015), where BRDC is the result of interaction between pathogen, host and environmental factors. Environmental conditions include crowding in pens, inclement weather, inadequate ventilation, poor colostrum quality and poor nutrition. Many of the *M. bovis* calf pneumonia reports are from feedlots or housed calves, where mortality rates can be high (Gagea et al., 2006). Experimentally induced *M. bovis*-associated respiratory infections were considered to induce more moderate pneumonic lesions than natural infections, where an exudative bronchopneumonia has been described with associated areas of coagulative necrosis (Rodriguez et al., 1996).

2.4.5 Keratoconjunctivitis

Keratoconjunctivitis (pinkeye), is usually characterised by bilateral conjunctivitis, photophobia, tear staining and keratitis. *Moraxella* spp. and some *Mycoplasma* spp., including *M. bovoculi*, are often isolated from ocular swabs taken from clinically affected eyes (Rosenbusch, 1985). *Mycoplasma bovis* has been associated with a pinkeye outbreak in Israel where *M. bovoculi* and *M. bovis* were both isolated from calves, but where no *Moraxella* spp. or other non-mycoplasma bacteria were identified. It was surmised the keratoconjunctivitis was secondary to respiratory disease in these calves, which was possibly due in part to BRSV and *M. bovis* infection (Levisohn et al., 2004).

2.4.6 Arthritis

Mycoplasma bovis arthritis can affect all ages of cattle, especially calves (Maunsell & Donovan, 2009). Arthritis is often a concurrent disease in cases of mastitis or pneumonia at an animal or herd level (González et al., 1993; Stipkovits et al., 1993). Clinically affected animals present with a septic arthritis, which is usually non-weight bearing and with joint swelling and pain. Pyrexia may be present (Stipkovits et al., 2000) and large rotator joints, including the shoulder, elbow, carpus, hip, stifle and hock (tarsus), are most commonly involved (Gagea et al., 2006; Pfützner & Sachse, 1996). Similar to

mastitis and respiratory disease, arthritis caused by *M. bovis* infection responds poorly to antimicrobial therapy (Romváry et al., 1977).

2.5 Diagnostic Techniques

2.5.1 Microbiology

The traditional detection of viable *Mycoplasma* spp. has been by culture, and is recognised as the 'gold standard' for the diagnosis of *M. bovis* disease, including mastitis (Murai et al., 2014). However, more recently, PCR technology has been used, as it offers more advantages and fewer limitations compared to culture (Parker et al., 2018).

To prevent bacterial culture false negatives, care needs to be taken at each step of sampling, handling, storage and laboratory processing, where complex media, specific equipment and a high technical skill level is required (Boonyayatra et al., 2010). The intermittent shedding of *M. bovis* in mastitic cows, especially chronic and subclinically infected cows, can also cause false negatives (Biddle et al., 2003).

Milk samples for culture need to be collected aseptically and kept cool, with some researchers ice packing samples prior to dispatch to the laboratory (Biddle et al., 2005). While low cost, culturing is also limited by Mycoplasmas' inability to proliferate in milk; they can also be overgrown by other bacteria (Parker, House, Hazelton, Bosward, & Sheehy, 2017). The specific methodologies with respect to enrichment medias used, incubation temperatures, atmospheric conditions (% CO_2) present and time incubated for, differed between researchers (Radaelli et al., 2011; Vähänikkilä et al., 2019).

Mycoplasma spp. bacterial colonies have a classic 'fried egg' appearance (Parker et al., 2018). All mycoplasma isolates need to be speciated by either antibody tests, namely immunofluorescence or indirect immunoperoxidase, or PCR (Imada et al., 1987).

2.5.2 Molecular Diagnostics

2.5.2.1 Conventional PCR

PCR is often compared to culture methodologies. The important differences are (i) PCR does not need a viable organism but nondegraded DNA is still needed for amplification (ii) PCR has faster turnaround time, which is valuable in making a clinical diagnosis and (iii) PCR can differentiate between *Mycoplasma* spp. (Parker et al., 2018). Conventional PCR detects the presence of the pathogen but does not quantify pathogen load. A further post-PCR step is needed for detection or visualisation of

the DNA. This outcome is achieved through denaturing gradient gel electrophoresis (DGGE), where amplified DNA is visualised as bands (McAuliffe et al., 2005).

Since PCR technology was developed in the mid-1980s there has been further development of different target genes as primers for *M. bovis* detection. The 16S rRNA gene, part of the 30S small subunit of a bacteria ribosome, has a slow rate of evolution, is highly conserved, and is widely used in bacterial species identification, including *M. bovis*. This gene is also used in phylogenetic studies as a reliable 'molecular clock'. The targeting of the 16S rRNA gene offers adequate specificity of *M. bovis* against most *Mycoplasma* spp., except *M. agalactiae* (González et al., 1995). The 16S-23S rRNA gene region has been used as a target for multiple species detection, specifically to discriminate between other *Mycoplasma* spp. and *Acholeplasma* spp., which are contaminants (Tang et al., 2000). A further development is the use of targeting the *M. bovis uvrC* together with *Mycoplasma* spp./*Acholeplasma* spp. 16S rDNA genes, in a conventional multiplex PCR, to allow identification of seven *Mycoplasma* spp. and two *Acholeplasma* spp. (Gioia et al., 2016).

2.5.2.2 *Real-Time PCR (qPCR)*

Technology improvements saw the development of qPCR. This method differs from reverse transcription PCR or RT-PCR. Unlike conventional PCR, which needs DGGE as a post-PCR step, qPCR is quantitative and measures the amplification as it occurs.

Similarly to conventional PCR, to increase specificity and decrease cross-amplification with other *Mycoplasma* spp., alternative genes to the 16S rRNA gene of *M. bovis* have been investigated (Cai et al., 2005). The *M. bovis uvrC* gene is highly stable and does not cross amplify with non-*M. bovis* species (Thomas et al., 2004). The oppD/F gene (Cai et al., 2005), involved with cell membrane transport (Orchard & Goodrich-Blair, 2004), and fusA gene have also been targeted (Boonyayatra et al., 2012). Each target gene has a reported qPCR detection limit. The *uvrC* gene for milk is 2.4×10^2 cfu/ml and the oppD/F gene is as low as 1×10^2 cfu/ml.

Real time PCR multiplex assays for the detection of multiple *Mycoplasma* spp. at one time (i.e. *M. bovis*, *M. bovigenitalium* and *M. californicum*) have also been developed (Parker, House, Hazelton, Bosward, & Sheehy, 2017). Similarly, DNA mastitis pathogen multiplex assays are also available for other common mastitis pathogens (e.g. *S. aureus, S. uberis and S. agalactiae*) (Gillespie & Oliver, 2005). An important limitation of PCR methods in the detection of *M. bovis* is that while the bacteria does not need to be viable, the animal still needs to be shedding the bacteria.

There are numerous commercially available qPCR testing kits for *Mycoplasma* spp. (Parker et al., 2018). These include (i) PathoProof assays by Thermo Fisher Scientific (Australia) (ii) Mastitis HP3 PCR

by QIAGEN (Australia) (iii) Bovicheck *M. bovis* PCR kit by Biovet Inc (Canada) (iv) Mastit4 assays by DNA Diagnostics A/S (Denmark). In NZ, VetMax™ *M. bovis* kit by TaqVet (France) has been used. Australian work reported an estimated specificity at the herd level of 97-100% for *M. bovis* detection using the PathoProof PCR kit (Penry et al., 2014). Danish work used a Major-3 PathoProof kit to perform qPCR testing on BTM (Nielsen et al., 2015) while Estonian work used the Mastit4B kits (Timonen et al., 2017).

2.5.3 Immunodiagnostics

2.5.3.1 *Development of ELISA for M. bovis*

Development of an *M. bovis* ELISA that is both sensitive and specific has involved the study of many different proteins (Behrens et al., 1994; Robino et al., 2005). Australian researchers (Wawegama et al., 2014) isolated an immunogenic protein, mycoplasma immunogenic lipase A (milA), and a recombinant fragment of this milA protein was used to develop both an indirect IgM and IgG ELISA. The IgG ELISA had an epidemiological sensitivity (Se) and specificity (Sp) of 92.9% and 98.7% respectively. This ELISA is now commercially available, as ID.vet Screen *M. bovis* Indirect, to measure *M. bovis* antibodies in serum and milk. Immunodiagnostic techniques indicate the cow or herd is currently infected or at one time has been infected, or alternatively vaccinated against *M. bovis* (Fox et al., 2003; Nicholas & Ayling, 2003).

2.5.3.2 *Seroconversion*

Seroconversion is both the development of antibodies to an infectious organism in response to experimental or natural infection, or the administration of a vaccine and a change from a seronegative to a seropositive condition. Cows with experimentally induced *M. bovis* CM produced a B cell humoral antibody response, which can be measured in milk, serum, and blood. This was historically measured using the indirect haemagglutination (IHA) technique (Bennett, 1978; Bennett & Jasper, 1977b; Cho et al., 1976). A T cell cell-mediated immune (CMI) response also developed.

Development of Antibodies

Experimental vaccination work found that following exposure to *M. bovis* antigens seroconversion took two to three weeks, as measured by an IgG ELISA (Nicholas et al., 2002). Wawegama et al. (2014) experimentally infected calves with *M. bovis* and reported calves seroconverting by 21-24 days post infection based on an IgG ELISA and a cut point of OD% ≥47. However, the detection of antibodies relative to onset of disease under field conditions has produced varying results.

Brys et al. (1992) noted with an early 'in-house' laboratory ELISA, *M. bovis* titres developed 10-14 days after disease onset, and hence infection could not be detected within the incubation period (Pfützner & Sachse, 1996). French work with naturally exposed animals, using a VspA-based ELISA, concluded titres of individual animals were not well correlated with infection or disease, and some diseased animals did not develop high titres (Le Grand et al., 2002). In contrast, Danish work (Petersen et al., 2018), which measured serum ELISA titres of *M. bovis* CM cows with the BioX Bio K 302 kit and used cut point of ODC% ≥37, showed, with the exception of one cow (from a four herd study with 18 likely *M. bovis*-associated mastitis cows) serum ELISA titres at the day of disease onset were above the recommended cut off. Important findings were there was marked variability in antibody response between cows and on an individual cow level, the antibody response was dynamic. Recent Australian work, using the same kit and cutpoint %, found that of 16 naturally infected *M. bovis* milk qPCR positive CM cows, 15 cows were serum ELISA positive 7-13 days after the individual diagnosis of each cow. This finding suggests that time since potential infection needs to be considered when interpreting serum ELISA results (Hazelton et al., 2018).

Persistence or Longevity of Antibodies

Consensus in the literature on the persistence or longevity of antibodies to *M. bovis* is lacking. Animal level experimental work showed IgA and IgG (milk whey IHA) remained raised for at least 57 days (Bennett & Jasper, 1980). Danish work (Petersen et al., 2018) agreed with this finding and noted a decline in serum antibody level within two months from clinical disease onset. In an Irish outbreak (Byrne et al., 2000), the cows were ELISA positive 27 weeks post start of the outbreak after no clinical cases had been diagnosed for 15 weeks. In this study an ELISA technique standardised by Liberal in 1988 using *M. bovis* sonicated antigen was used. Recent Finnish work (Vähänikkilä et al., 2019) that investigated 17 farms that had confirmed cases of *M. bovis* CM found milA ELISA-detectable antibodies persisted in cow serum for at least 1.5 years. This finding was irrespective of farm infection status, SCM or CM. Of interest, the Danish work used an ELISA assay kit (BioX Bio K 302 kit) that has a lower sensitivity compared to the milA ELISA kit (Petersen et al., 2018; Wawegama et al., 2016). The literature suggests that there are major gaps in our understanding of seroconversion and antibody persistence subsequent to *M. bovis* infection in a herd (Hazelton et al., 2018; Vähänikkilä et al., 2019).

2.5.4 Immunohistochemistry

A definitive diagnosis of disease due to *M. bovis* infection is reliant on being able to demonstrate the pathogen in diseased tissue. Immunohistochemistry (IHC) enables the presence, location, and distribution of *M. bovis* in diseased tissue to be determined (Maunsell et al., 2011). Using PM samples and then the process of formalin fixation followed by paraffin-embedding, *M. bovis* antigen can be

detected in situ by IHC (D. Adegboye et al., 1995; Gagea et al., 2006). Immunohistochemistry, the most common application of immunostaining, is a technique whereby enzyme-conjugated antibodies catalyse a reaction, causing a colour change, and specific antigens (i.e. *M. bovis*) can be localised within a tissue.

Immunohistochemistry has been used in lung lesions from Canadian feedlot calves, where *M. bovis*, amongst other pneumonia-causing bacteria and BVDV have been found and their roles in lesion development have been investigated (Gagea et al., 2006). Immunohistochemistry on *M. bovis* infected lungs and joint tissue has also been used in qPCR and culture validation studies (Clothier et al., 2010). However, there are limited reports in the literature of IHC being used to detect *M. bovis* in cases of CM. Two mastitic cows from a small herd in northern Italy had mammary tissues IHC stained to show abundant, brown staining *M. bovis* antigen in the necrotic debris around mammary ducts (Radaelli et al., 2011), strongly suggesting that *M. bovis* had a primary role in the development of these mammary lesions.

2.6 Sensitivity and Specificity of Tests

The interpretation of the results of diagnostic tests needs to be made with the knowledge of diagnostic (or epidemiological) sensitivity (Se) and specificity (Sp), and an understanding of analytical Se and Sp. Without this, there is potential for the researcher to publish incorrect results. For example, the 1997 prevalence of *M. bovis* in Australian dairy cows being initially overstated (Ghadersohi et al., 1999), but later corrected (Morton et al., 2014).

The analytical parameters can be considered descriptions of a laboratory's assay ability to detect small amounts of a target pathogen (sensitivity) and to react only when the pathogen is present (specificity). Analytical Se and Sp are components of diagnostic Se and Sp. Diagnostic Se however, is the proportion of infected animals or herds that test positive and specificity is the proportion of uninfected animals or herds that test negative (Sergeant & Perkins, 2015a). Diagnostic Se and Sp can be reported at an individual animal or group level.

By using 'gold standard' samples i.e. individual cow CMS cultures (Murai et al., 2014), from known uninfected and infected farms, an estimation of diagnostic Se and Sp can be made (Morton et al., 2014). However, latent class analysis (LCA) also allows the estimation of diagnostic Se and Sp by using results from animals in populations with different disease prevalences (Enøe et al., 2000; Hui & Walter, 1980; Nielsen et al., 2015) even when a 'gold standard' test is not available (Toft et al., 2005).

2.7 Pathology of *Mycoplasma bovis* Mastitis

The gross lesions and histopathological findings from cases of *M. bovis* CM are not widely reported in the literature. Earlier reports are primarily based around experimentally induced disease (Bennett & Jasper, 1977a; Seffner & Pfützner, 1980; van der Molen & Grootenhuis, 1979). The findings of a small number of natural infections are briefly reported as part of case summaries (Al-Abdullah & Fadl, 2006; Pothmann et al., 2015; Radaelli et al., 2011).

2.7.1 Gross Lesions

The clinical and gross changes in the experimentally infected udder over the first 12 days post infection are well documented (van der Molen & Grootenhuis, 1979); infected quarters progress from having a diffuse soft swelling, to marked swelling and then udder induration within the 12 days, with atrophy and fibrosis of the affected gland (Seffner & Pfützner, 1980). The characteristic mastitis in experimentally infected cows was described as fibrinosuppurative to caseonrecrotic (Bennett & Jasper, 1977a). An increase in fibrous tissue, which may give the cut surface a yellow to grey appearance, makes infected udders hard to cut into (Hale et al., 1962; Jasper, 1981a). Grossly, abscesses as large as 5-10 cm in diameter were also noted, as were enlarged supramammary lymph nodes (Bennett & Jasper, 1977a).

The gross lesions present in the mammary gland of three affected cows are described in an Italian study of 45 clinically affected cows (Radaelli et al., 2011). Chronically affected mammary glands became agalactic and indurated. Milk separated into a floccular precipitate and watery supernatant. Nodular lesions with a necrosuppurative exudate and parenchymal fibrosis were noted. Enlarged supramammary and milk ducts with seropurulent exudate were also reported in naturally infected cows (Byrne et al., 1998). Unfortunately, two other studies (Al-Abdullah & Fadl, 2006; Pothmann et al., 2015) reported brief clinical signs similar to other natural infections, but not gross findings on PM.

2.7.2 Histopathology

A comprehensive review of histological changes from cows experimentally infected with *M. bovis* is included in the literature (Bennett & Jasper, 1977a; van der Molen & Grootenhuis, 1979). Infected cows were sampled on specific days post infection through to the time of slaughter. Acutely, the inflammatory response was mainly granulocytes (eosinophils and neutrophils) within alveoli. This response progressed to an interstitial-type reaction with eosinophils and mononuclear cells, which included both lymphocytes and plasma cells. The chronic stage, which was only 12 days post infection, saw the alveoli and ductules with progressive fibroplasia. In another study describing chronic lesions,

granulomata, epithelial hyperplasia, and some reticuloendothelial cells with fat in their lumens were also noted (Hale et al., 1962). Importantly, microscopic abscesses that contained viable *M. bovis* were also identified (Bennett & Jasper, 1977a), suggesting chronically infected cows could be an ongoing source of infection in infected herds.

Histology reports from clinical cases investigated as part of observational studies contain similar lesion descriptions. A pyogranulomatous galactophoritis with marked fibrosis has been described (Pothmann et al., 2015; Radaelli et al., 2011). Radaelli et al. (2011)also noted areas of 'severe multifocal to coalescing necrosuppuration.' A range of changes similar to those changes were also found in cows in a Saudi Arabian outbreak (Al-Abdullah & Fadl, 2006). As the mastitis became more chronic there was tissue fibrosis in the interacinar and interlobular areas, which was associated with marked atrophy of the gland.

2.8 Control and Eradication

Many strategies may be employed as part of infectious disease control and eradication efforts. The strategies of test and segregate, test, and slaughter, vaccinate, and administration of antimicrobial therapy in response to outbreaks of *M. bovis* CM are considered. There is no one standard approach to managing an outbreak of *M. bovis* CM, and the aim of the management plan i.e. control, elimination or eradication may determine strategies used (Sergeant & Perkins, 2015c). However, an attempt to cull all cows with mycoplasma IMI should be strongly recommended (Maunsell et al., 2011).

2.8.1 Test and Segregate/Test and Slaughter

Diagnostic tests available for application at the herd and cow level to aid in the diagnosis of an *M. bovis* mastitis outbreak include: BTM qPCR, ELISA, and culture; and cow-level serum ELISA, qPCR and culture on QMSs or CMSs, and qPCR on palatine tonsil swabs. Results from diagnostic tests combined with monitoring for clinical signs enables decisions about segregation and culling. From a management perspective, Parker et al. (2018) recommends that any cow suspected of being clinically infected with *M. bovis* while waiting for a test result should be segregated into a third herd that is distinct from the main milking herd and the red hospital herd.

It is possible to eliminate *M. bovis* mastitis from a dairy herd through use of a combination of test and slaughter, ongoing surveillance, and closing of the herd to cattle introductions (Maunsell et al., 2011). This finding is supported by other American work (González & Wilson, 2003) and Irish studies (Byrne

et al., 1998). A closed herd is required for long-term success. Eradication from individual herds has occurred for Dutch herds (Wentink et al., 1987) and small Swiss herds (Aebi et al., 2015).

However, some researchers consider that *M. bovis* mastitis (at the herd level) is a self-limiting disease (Nicholas et al., 2016). Spontaneous elimination, by an unknown mechanism, sees most mastitis outbreaks resolve within the first two months (Jasper, 1981b), and other outbreaks within one year (Fox et al., 2003) although some outbreaks may continue for a year or more (Bayoumi et al., 1988; Bicknell et al., 1983). An American study (Punyapornwithaya et al., 2012) designed to investigate control strategies found within a month, 14 of 18 herds had cleared a mastitis outbreak – with or without culling. Therefore, there is some evidence that partial herd culling based on clinical signs or test results is not an effective control measure. Nevertheless, the infection cycle must have been broken in some way, for herds to clear a mastitis outbreak (Maunsell et al., 2011; Nicholas et al., 2016). However, the terms self-limiting, spontaneous elimination, and clearance of infection are not well defined in the literature. It would therefore be difficult to claim successful elimination of *M. bovis* infection from a herd, if only targeting animals that have CM.

2.8.2 Vaccination

A detailed discussion of vaccines used for *M. bovis*-associated diseases is outside the scope of this review. Much of the vaccine technology has been focussed on the development of a vaccine for calf pneumonia. Over time different adjuvants (e.g. formalin and saponin), and different types of vaccines made from *M. bovis* membrane proteins have been trialled. Many of the vaccines for respiratory disease were multivalent and combined other mycoplasma species and other bacterial and viral pathogens (e.g. *M. dispar*, BPI-3, *Mannheimia haemolytica*) (Maunsell et al., 2011; Nicholas et al., 2002).

Promising challenge studies for vaccines have largely preceded ineffective field trials (Maunsell et al., 2011). In some cases vaccines have been found to exacerbate clinical signs of disease (e.g. a respiratory vaccine worsened lung pathology (Nicholas et al., 2002) and a specific *M. bovis* bacterin, which was inoculated via the intramammary route, increased the severity of mastitis (Boothby et al., 1986)). There is no published literature to show that current vaccines avert, reduce the incidence of, or improve the clinical signs of mastitis due to *M. bovis* (González & Wilson, 2003). The adaptive immune responses of *M. bovis* may also contribute to the fact that attempts to develop effective vaccines against *M. bovis* have so far been unsuccessful (Fox, 2012; Maunsell et al., 2011).

2.8.3 Antimicrobial Therapy

The control of *M. bovis*-associated diseases, especially CM, with antimicrobial therapy is largely unsatisfactory as disease is often refractory to treatment (Ayling et al., 2014; Calcutt et al., 2018; Nicholas et al., 2016) with some researchers even recommending not treating *M. bovis* CM. Their philosophy is cows that have spontaneous resolution of CM can become intermittent, subclinical shedders and therefore should be considered permanently infected (Maunsell et al., 2011). Others consider, based on field experience, antimicrobial therapy against mycoplasmas is not economically sustainable (Nicholas & Ayling, 2003).

Mycoplasmas have several characteristics that enhance their resistance to antibiotics, including the lack of a cell wall. Bactericidal β-lactam antibiotics are known to work by preventing the synthesis of the peptidoglycan layer of the cell wall. Hence, mycoplasmas and importantly *M. bovis*, are fundamentally resistant to penicillins and cephalosporins. Similarly, mycoplasmas cannot synthesize folic acid and so are also resistant to sulphonamides (Maunsell et al., 2011). On pharmacological first principles, mycoplasmas should be susceptible to antimicrobials that interfere with DNA synthesis (e.g. fluroquinolones) and protein synthesis (e.g. tetracyclines, macrolides, lincosamides, chloramphenicol and its analogues). However, there is an increasing body of work to show there is increasing *in vitro* resistance to several, if not all, the main classes of antimicrobials (Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016), including tetracyclines, macrolides, fluroquinolones and spectinomycin, and aminocyclitol (Calcutt et al., 2018; Pothmann et al., 2015). *In vitro* antimicrobial sensitivity testing is presently the best estimate of antimicrobial sensitivity and potential *in vivo* effectiveness (Ayling et al., 2014).

While pharmacological studies encompass the spectrum of *M. bovis* diseases, specific reference to *M. bovis* mastitis isolates in a UK study (Ayling et al., 2000), when compared to respiratory isolates, showed minimum inhibitory concentrations (MIC) values for 12 antimicrobials were high. The exception was the MIC values for the lincosamides (lincomycin and clindamycin) for respiratory isolates, which were low and had not changed over the time periods the study took place. However, the MIC values for the mastitis isolates for the lincosamides were suggestive that resistance was also developing to this class of drugs.

Mycoplasmas' ability to target different organ systems assists their survival in the host as therapeutic MICs may not be reached (Nicholas & Ayling, 2003). Furthermore, the intracellular sequestration of mycoplasmas and its ability to form biofilms may also enable the organism to elude antimicrobial therapy (McAuliffe et al., 2006).

2.9 Economic Cost of *M. bovis*

Mycoplasma bovis disease due to the spectrum of clinical disease syndromes, lack of response to antibiotics, chronicity of disease, production losses, and potential morbidity and mortality can be devastating financially to farmers whose herds become infected (Maunsell & Donovan, 2009). While there are numerous estimates on economic costs to the farmers and production indices of different countries, Australian Dr. Glenn Browning recently stated in London, 'the full economic cost of *M. bovis* in cattle production is yet to be determined' (Chalker, 2019). The economic cost of animal welfare has not been factored into many of the international costings of *M. bovis* disease.

M. bovis-associated respiratory disease alone, across the UK and Europe, has been estimated at a cost of €576 million annually (Nicholas & Ayling, 2003). Twenty years ago, the cost to the American dairy industry, due to mastitis alone, was estimated at nearly $USD 110 million annually (Rosengarten & Citti, 1999). The true economic cost of *M. bovis* CM to the UK is not known, in part because it assumed that some of the 10% of mastitis cases that go undiagnosed in the UK each year are caused by *M. bovis* infection (Ayling et al., 2004).

The NZ Government has economically analysed the potential cost of *M. bovis* to NZ. This analysis in part underpinned their decision on 'Planned Eradication' of *M. bovis* (Biosecurity New Zealand, 2018a; New Zealand Government, 2018). Estimates associated to a few scenarios have been published. The full cost of phased eradication over 10 years was estimated at 886 million NZD. This amount was split into 16 million NZD on loss of production and 870 NZD million on the cost of the Programme, including farmer compensation. The option of long-term management of this disease pushed the economic costs out to 1.2 billion NZD. To not respond at all resulted in a projected 10-year cost of 1.3 billion NZD, with the farming sector enduring ongoing productivity losses.

Chapter 3 Materials and Methods

3.1 Background to Study

National Surveillance Testing

As part of a national surveillance programme for *M. bovis*, bulk milk testing (BMT) of all NZ dairy farms commenced in March 2018. For the 2018/19 dairy season, spring bulk tank milk (BTM) samples were collected on every NZ dairy farm supplying milk for commercial processing, approximately four weeks after the planned start of calving (PSC), and repeated then again, every two weeks for a 12-week period on six occasions. Each sample was tested by qPCR, and alternate samples were tested by ELISA. Any potential Infected Properties (IPs) (3.4.3.1 Case Definitions), which were identified from the routine BTM surveillance testing programme, would then go through an increased series of testing, which included both BTM and individual cow serum ELISA (Ministry for Primary Industries, 2018). As part of the decision to eradicate *M. bovis* from NZ, all stock from IP's were to be slaughtered (New Zealand Government, 2018.)

For the purpose of this study, BTM surveillance testing, on-farm surveillance testing and slaughter surveillance testing will be referred to as the *M. bovis* Programme or Programme testing for short.

First Tentative Diagnosis of M. bovis on Farming Operation in Study

On 10.9.2018, the North Otago (NO) dairy farm owners were informed by MPI, that one of their four NO dairy farms, had an *M. bovis* detection by BTM qPCR test, pending further testing for confirmation of *M. bovis*. The BTM sample had been collected on one property, identified as Farm 1 in this study, on 30.8.2018.

3.2 Enterprise Background

3.2.1 Outline of Farming Operation

The large scale, family owned, multi-farm dairy business consisted of four seasonal-calving, pasture - based, irrigated dairy farms (Farms 1 to 4), one leased grazing block (Farm 5), and one family owned run off property (Farm 6). Young replacement stock was grazed at another grazing property (Farm 7), which was located outside of NO. See Fig. 3-1 for farm locations. The dairy farms, except Farm 4, were located in close proximity to one another inland from Oamaru, NO. Farm 4 was located approximately 40 km further inland. The farming operation has dealt with the same veterinary practice, Veterinary Centre Oamaru for over 25 years and the author of this manuscript (KPK) has been the primary clinical veterinarian for the last 10 years.

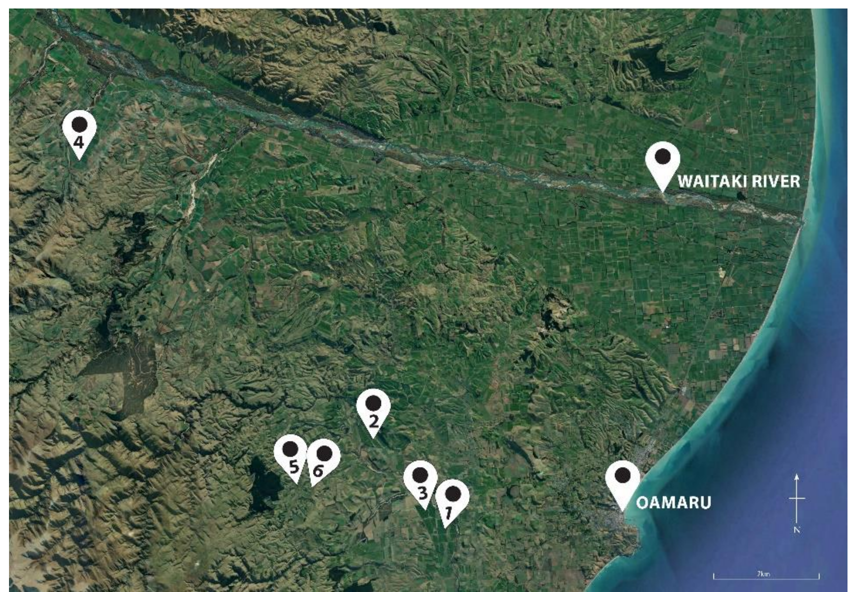


Figure 3-1 Map of North Otago showing location of study farms

Source of map. Google. (n.d.). [Google map of North Otago]. Retrieved 18.8.20 from *https://www.google.co.nz/maps/place/Otago/@-45.0265847,170.8245145,38962m/data=!3m1!1e3!4m5!3m4!1s0xa82b10750e63ed15:0x6042a57e4192feb7!8m2!3d-45.4790671!4d170.1547567?hl=en*

The four dairy farms operated as System 4 farms under the DairyNZ definition of production systems where imported feed is used all year, throughout lactation and for dry cows (DairyNZ, 2017b). This dairy business was highly integrated with frequent stock movements between the four dairy farms, run-off, and both grazers. Key performance indicators (KPIs) for the four dairy farms are detailed in

Table 3-1. This dairy operation, unlike most NZ seasonal dairy farms, dried off cows in 5 batches according to calving date with all cows having a minimum of a 60-day dry period, rather than on a single or small number of dates as is commonplace. In the 2017/18 season, cows were dried off at approximately two-week intervals from 25.5.2018 to 20.7.2018. The last dry off effectively occurred just prior to the PSC of the 2018/19 calving period, 30.7.2018.

Table 3-1 Key Performance Indicators and test results for the dairy farming enterprise described in this study

		Farm 1	Farm 2	Farm 3	Farm 4
1	Number of cows (Sept 2018)	1050	1000	705	630
2	Total land area (ha)	308	322	197	265
3	Effect ve Land Area (ha)	287	287	171	150
4	Stocking Rate (cows/ha)	3.6	3.5	4.1	4.2
5	Total Production kgMS 2017/18	489,676	511,163	355,404	245,724
6	KgMS/cow (2017/18)	483	513	501	500
7	KgMS/eff ha	1706	1781	1804	1638
8	Supplement feed/cow/year (kgDM)	1450	1450	1450	1800
10	Breed (% Friesian) [3]	86%	67%	71%	75%
11	Number of Participant codes in herd [3]	118	73	77	84
12	Topography of Farms	Flat	Flat to rolling hill	Flat	Flat to hill
13	Management Structure	Manager	Manager	LOSM [a]	LOSM
14	Shed Design	Rotary with ACRs [b]	Herringbone	Herringbone	Rotary
15	6 Week In calf rate (target 78%) [1]	57%	56%	61%	51%
16	Calving Pattern 3 wks (target 67%) [1]	57%	53%	55%	57%
17	Calving Pattern 6 wks (target 88%) [1]	80%	75%	79%	79%
18	Calving Pattern 9 wks (target 98%) [1]	97%	91%	94%	93%
19	Average BMSCC [c] 2017/18	214,000	232,000	232,000	259,000
20	Average BMSCC 2018/19 (until depop.)	186,000	185,000	225,000	209,000
21	BCS Pre-calving July 2018 [4]	4.9	4.9	4.9	
	BCS Heifers Pre-calving July 2018 [4]		5.6	5.6	
22	BTM BVD Ag PCR [d] Spring 2017 [5]	Negative	Negative	Positive	Negative
23	BTM BVDAg PCR Spring 2018 [5]	Negative	Negative	Negative	Negative
24	Blood Profiles (n = 10 cows, day 3 post calving, July 2018) [6]				

	Units	Ref Range	Farm 1	Farm 2 Mean	Farm 2 Range	Farm 3 Mean	Farm 3 Range	Farm 4
Serum Mg	mmol/L	0.59-1.08		1.1	0.77-1.66	1.23	0.99-2.29	
Serum Ca	mmol/L	2-2.7		2.29	1.49-3.04	2.29	0.69-2.57	
Serum NEFA [e]	mmol/L	≤0.7 [2] with <30% above this		1.00	0.6-1.4	0.85	0.3-1.4	
Serum B12	pmol/L	150-1000		512	410-670	308	230-380	
Serum Se	nmol/L	140-2000		838	550-1010	852	640-1030	
Serum Cu	umol/L	7-20		13.3	11-15	9.5	7-13	

References
[1] (DairyNZ, 2017a)
[2] (Huzzey et al., 2012)

Data Sources
[3] MINDA ®
[4] Infovet
[5] LIC
[6] Vision VPT ™

Abbreviations
[a] LOSM = Lower order sharemilker
[b] ACR = Automatic cup removers
[c] BMSCC = Bulk milk somatic cell count
[d] BTM BVD Ag PCR = Bulk tank milk bovine viral diarrhoea antigen polymerase chain reaction
[e] NEFA = Non-esterified fatty acid

3.2.2 Aspects of Enterprise History

Mastitis History

Over the last five seasons, the farming enterprise has had a history of chronic mastitis cases. In a CM investigation late in the 2017/18 season on Farm 2, *S. aureus* was detected using bacterial culture and MALDI-TOF technology (matrix-assisted laser desorption/ionisation – time of flight mass spectrometry) in 24 of the 26 cows presented for testing because of recent apparent treatment failures (ATF) for CM. Thirteen of the 26 cows had *S. aureus* detected in multiple quarters. On-farm mastitis control policies, coupled with reproductive performance that was consistently lower than the national targets across this farming operation, limited the ability of the owners to cull cows with known mastitis problems.

Stock Trading

This farming operation had a history of trading dairy stock, and the 2016-born heifer calves were the first heifer replacements kept for a number of years. Previously, heifer calves were sold at weaning, and lines of in-calf heifers were purchased each winter as replacements. Farm 3 had a history of BVD being introduced into the farm in July 2017 from a line of purchased in-calf heifers. A hunt for persistently infected (PI) animals found a number of PI heifers, which were subsequently removed from the herd. By comparison, from 163 herds in the Waitaki/North Otago area (LIC supplied data. Animal Evaluation Unit, *personal communication – email 20.2.2020)* the median number of participant codes or birth herds in each current herd was 15, and 25% and 75% interquartile ranges were 5 and 33 respectively. The 95 percentile was 77 codes. Whereas, the number of participant codes for Farms 1 to 4 in this study, were 118, 73, 77 and 84 respectively.

First Mycoplasma bovis Test

With knowledge of the CM history of these four farms, when *M. bovis* was first diagnosed in NZ in July 2017, these farms were immediately put forward for voluntary testing, under the Programme. *Mycoplasma bovis* qPCR testing across these four farms in August 2017, which included BTM, colostrum, discard milk and a small number of CM cows (on Farm 1), were all negative.

3.3 Samples Collected from Farms and Selection of Animals for Testing

During the study period from 30.8.2018 when the first Programme BTM tests were collected, until the last cows were destocked from the three dairy farms (Farms 1, 2 and 3) on 19.2.2019, a wide range of samples were collected through the outbreak. The methodology of sample collection is described in 3.7. Table 3-2 summaries the herd-, animal-, and quarter-level testing carried out across the enterprise. Table 3-3 reports the dates of the four vet visits, under Programme directive, by the author (KPK) to Farms 1 and 2 for sampling of famer-selected CM cows. The diagnostic test cut points with Programme estimates of performance are summarised in Table 3-4.

The samples collected from farms are detailed in three parts. Part 1 reports the Outbreak Investigation at a herd-, animal- and quarter-level, Part 2 the Agreement Study and Part 3 the Pathological Findings.

Part 1 Outbreak Investigation

3.3.1 Herd-level Sampling

Bulk Tank Milk samples, for qPCR and ELISA, were collected from each farm as part of the Programme surveillance (in 3.1 Background to study).

3.3.2 Animal-level Sampling

3.3.2.1 Programme Surveillance

At the initial Programme surveillance visit to each farm, a selection of cows were blood tested for *M. bovis* serum ELISA and a CMS collected for *M. bovis* qPCR. A selection of calves were nasal swabbed, for *M. bovis* qPCR. The type of samples collected at subsequent Programme surveillance visits, and clinical visits, varied, depending on the specific information being sought. (Retrieved 16.2.20 from https://www.mpi.govt.nz/protection-and-response/mycoplasma-bovis/what-is-mpi-doing/testing-regime).

3.3.2.2 Vet Visits for Clinical Mastitis Cows

There were four vet visits for sampling of farmer-selected CM cows, Farm 1 26.9.2018 and 23.11.2018 and Farm 2 17.10.2018 and 28.11.2018. These cows were blood tested for *M. bovis* serum ELISA (except Farm 1 26.9.18), palatine tonsils swabbed and QMSed for *M. bovis* qPCR. While in the main on Farm 1, the cows presented were ATF cases (3.4.3.2), Farm 2 did draft some recent CM cases, still

receiving their first course of mastitis treatment, into the sampling group. Some cows were presented at the two visits on a farm and allowed a second sample collection.

Forty-four CM cows from Farm 1 on 23.11.2018 and 37 CM cows from Farm 2 on 28.11.2018, were also blood tested for BVD PCR Ag and Ab ELISA.

3.3.2.3 *Non-clinical Cow Prospective Cohort Study (see 3.11/6.1.4) on Farm 1 - Vet Visit*

For the visit on 10.1.2019, 133 cows were both blood tested for *M. bovis* serum ELISA and CMS for *M. bovis* qPCR.

3.3.2.4 *Slaughter*

At slaughter, Programme surveillance and CM cows were blood tested for *M. bovis* serum ELISA and palatine tonsil swabbed for *M. bovis* qPCR.

3.3.3 Quarter-level Sampling

3.3.3.1 *Vet Visits to CM Cows*

At the four vet visits to CM cows, QMS was carried out on mastitic quarters on farmer-selected cows, as recorded from farmer records.

3.3.3.2 *Farmer Collected*

Later in the outbreak from Farms 1, 2 and 3, again under Programme directive, the farmers collected QMS from CM cows.

Part 2 Agreement Study

3.3.4 Agreement Study

The animal-level sampling (serum ELISA and palatine tonsil swabs) and quarter-level testing (QMS sampling from vet visits to CM cows) for *M. bovis* CM cows were used to track *M. bovis* CM cows on-farm and at slaughter. Serum ELISA and palatine tonsil swabs were used to track non-clinical Programme surveillance cows at slaughter.

3.3.5 Pathology samples at slaughter

On 4.12.2018, mammary gland samples from 55 quarters, from 14 diagnosed *M. bovis* CM cows from Farm 1, were collected and examined at slaughter, by the author (KPK).

Table 3-2 Summary of herd-, animal-, quarter- level testing of Programme surveillance and vet diagnostic samples and tests taken during outbreak for Farms 1-4 on-farm and at slaughter. Unshaded is on-farm Programme surveillance

		Farm 1		Farm 2		Farm 3		Farm 4	
		Date	No. Samples	Date	No. Samples	Date	No. Samples	Date	No. Samples
Herd-Level	Bulk Tank Milk (surveillance at milk pick-up - tanker)	Between 30.8.2018 - 17.1.2019		Between 31.8.2018 - 7.1.2019		Between 30.8.2018 - 1.1.2019		Between 30.8.2018 - 4.3.2019	
		PCR	52	PCR	50	PCR	54	PCR	85
		ELISA	32	ELISA	29	ELISA	29	ELISA	7
Animal-Level (Programme surveillance)	Serum ELISA	18.9.2018	100	18.9.2018	100	19.9.2018	100	19.9.2018	100
		29.10.2018	10	7.1.2019	83	15.10.2018	100	12.10.2018	90
		10.1.2019	133	10.1.2019	54	24.10.2018	100	22.2.2019	100
		29.1.2019	82	11.1.2019	93	22.11.2018	200		
						1.2.2019	74		
	Palatine Tonsil Swabs qPCR	25.1.2019	100	7.1.2019	90	1.2.2019	94		
		29.1.2019	100	10.1.2019	90				
				11.1.2019	100				
	Milk CMS qPCR	18.9.2018	26	18.9.2018	25	19.9.2018	25	19.9.2018	25
		10.1.2019	133			15.10.2018	25	12.10.2018	26
						22.11.2018	32	20.2.2019	25
Animal-Level (Clinical mastitis cases)	Serum ELISA	23.11.2018	44	17.10.2018	27				
		4.12.2018	39	28.11.2018	36				
		17.12.2018	15	18.12.2018	25				
	Palatine Tonsil Swabs qPCR	26.9.2018	17	17.10.2018	26				
		29.10.2018	10	28.11.2018	36				
		23.11.2018	43	18.12.2018	25				
		4.12.2018	39						
		17.12.2018	15						
Quarter-Level (Clinical mastitis cases)	Milk QMS qPCR	26.9.2018	16	17.10.2018	27				
		23.11.2018	48	28.11.2018	42				
	Milk QMS qPCR (farmer collected)	5.12.2018	8	7.12.2018	7	21.11.2018	9		
		13.12.2018	5	24.12.2018	4	11.12.2018	6		
		18.12.2018	8	7.1.2019	5	13.12.2018	10		
		7.1.2019	7	9.1.2019	5	21.1.2019	7		
		8.1.2019	8	11.1.2019	3				
	Calf nasal swab qPCR	18.9.2018	39	18.9.2018	34	19.9.2018	40	19.9.2018	40
				26.9.2018	7	19.10.2018	40	12.10.2018	40
						22.11.2018	40		
	Calf serum ELISA	23.11.2018	74	30.11.2018	70	26.1.2019	100		

Table 3-3 Dates of vet visits to study Farms 1 and 2 for sampling and management of outbreak, and dates of slaughter of Mycoplasma bovis CM cows (number of cows)

	Clinical Visit 1	Clinical Visit 2	Clinical Visit 3	Clinical Visit 4	Slaughter of clinicals and nos.
Farm 1	26.9.2018 16 ATF cows sampled	24.10.2018 [1] 36 ATF cows inspected/palpated	22.11.2018 [1] 18 ATF cows inspected/palpated	23.11.2018 44 ATF cows sampled	4.12.2018 (26) [2] 17.12.2018 (15)
Farm 2	17.10.2018 27 ATF cows sampled	13.11.2018 [1] 30 ATF cows inspected/palpated	28.11.2018 36 ATF cows sampled		18.12.2018 (25)

[1] Three specific farmer-initiated vet visits, Farm 1 24.10.2018 and 22.11.2018 and Farm 2 13.11.2018 where management of the outbreak was further discussed and CM cows inspected and udders palpated.
[2] Mammary glands sampled for pathology at this visit

Key [shaded box] Vet diagnostic sampling of CM cows on farm

Table 3-4 Diagnostic test cut points with programme estimates of performances

	Cut Point for positive test result	Test Performance	
		Diagnostic Sensitivity	Diagnostic Specificity
BTM ELISA (ID.Vet ELISA)	SP% ≥ 30 [1]	47% [95% CI: 23-72%] [3]	98% [95% CI: 96-99%] [3]
Individual Animal Serum ELISA (ID.Vet ELISA)	SP% ≥ 60 [1]	89.3% [95% CI: 87.2-91.5%] [3]	95.0% [95% CI: 94.3-95.6%] [3]
BTM qPCR (VetMAX™)	CT <45 [2]	Not estimated, <40% [4,5]	Approaching 100%[2]
QMS qPCR (VetMAX™)	CT <45	Not estimated, <40% [4,5]	Approaching 100%[2]
Tonsil qPCR (VetMAX™)	CT <45	Estimated at ~38% [5]	Approaching 100%[2]

References

[1] (ID.vet, 2018)
[2] (Thermo Fisher Scientific, 2020)

[3] (Mackereth & Marquetoux, 2019)
[4] (Ministry for Primary Industries, 2019b)
[5] (Sawford, 2019)

3.4 Definitions Used in Case Study

3.4.1 Definition of Outbreak of M. bovis Clinical Mastitis

There are many definitions of an outbreak of disease (Sergeant & Perkins, 2015b) and specifically an outbreak of *M. bovis* mastitis (Petersen et al., 2018; Punyapornwithaya et al., 2010; Vähänikkilä et al., 2019). However, as *M. bovis* is an Unwanted Organism in NZ, the first confirmed *M. bovis* CM case on a farm, whether *M. bovis* was confirmed alone or in conjunction with other pathogens by milk qPCR, would constitute an outbreak of *M. bovis* CM under the NZ situation and this is the meaning of the term in this thesis.

3.4.2 Terms for M. bovis Mastitis

The three *M. bovis* mastitis definitions stated in 2.3.5 Prevalence will be used in this thesis. Namely *M. bovis* seropositivity, but not detected in milk does not represent an *M. bovis* IMI; secondly an *M. bovis* SCM, with IMI present; and thirdly an *M. bovis* CM case.

3.4.3 Case Definitions

Case definitions are described on both herd level, as used in the Programme, and then cow level for an *M. bovis* CM case.

3.4.3.1 Herd-level M. bovis (Programme) Confirmed Positive

(i) Bulk Tank Milk (BTM) qPCR detection, further processed by conventional PCR and sequenced at MPI's National Animal Health Laboratory (AHL), Wallaceville, Wellington to confirm *M. bovis* presence and hence herd *M. bovis* positivity. *(The methodology of confirmatory conventional PCR testing and sequencing is described in 3.8.2)*

or

(ii) Two rounds of on-farm surveillance are considered positive at a herd-level. At the time of this case study, a round of on-farm surveillance was considered positive when ≥ 10% of individual cow serum samples tested positive on ELISA.

Note: qPCR tests have been used widely in this study. For the reporting of qPCR tests, given the poor diagnostic sensitivity of qPCR tests in the detection of M. bovis, laboratory test results (Chapter 4) have generally been reported as 'Detect' or 'Not detect'. However, once confirmatory testing has been done on multiple samples, over numerous testing dates, on an IP, the terminology of M. bovis qPCR 'detection' and M. bovis 'positive' may become interchangeable for the study farms e.g. for Programme BTM qPCR tests reported CT results, if the sample was < 45, it was deemed 'positive'.

3.4.3.2 *Cow-level M. bovis CM Case for this Study (and Explanatory Notes 1-7)*

An individual cow was defined as having an *M. bovis* CM case when she was either:

(i) Milk qPCR positive from at least one gland, and a mastitis case with multiple courses of treatment (ATF)

or

(ii) Milk qPCR positive from at least one gland and a mastitis case with a single course of treatment

(1) A tentative diagnosis of a case of *M. bovis* CM was made from an individual cow on qPCR from a QMS, but similarly to a herd level diagnosis, was deemed positive after the sample was run through conventional PCR with the product being sequenced to *M. bovis*, at AHL. (All CM cows presented for sampling, where farmer-selected.)

(2) For inclusion as an *M. bovis* CM case, a cow needed:

(a) a milk positive qPCR from a gland and a history of antibiotic treatment in that gland.

(b) tag details to be correct and traceable e.g. radio frequency identification (RFID) tag linked correctly to lifetime identification (LID) tag, which then linked to farm management tag.

(c) to have CM during lactation.

Each of these criteria excluded one to two cows per farm due to gland treatment history not matching gland sampled; cows having multiple identifications or couldn't be traced; or sick/dried off cows subsequently developed mastitis and were presented for sampling, with no specific mastitis treatment record.

(3) The apparent treatment failure (ATF) of a mastitis case was defined when a gland received more than one course of antibiotic treatment, and where the treatment was separated by less than 30 days e.g. the same gland was re-treated within 30 days of first treatment (McDougall et al., 2007; McDougall et al., 2009).

(4) A single (extended) course of antibiotic treatment for mastitis was often given late in the outbreak e.g. in early December when farmers delivered milk samples to the clinic, as either a farm management decision based on likely cure or to safeguard against potential meat withholding periods (WHPs) at slaughter.

(5) Under these definitions, 48 cows from Farm 1 were considered as having *M. bovis* CM, with 37 under definition (i), multiple courses of treatment and an ATF and 11 under (ii), a mastitis case with a single course of treatment. For Farm 2, there were 42 cows, with 30 under definition (i) and 12 under (ii) respectively.

(6) The total of *M. bovis* CM cows on each farm includes the samples which the farmers delivered to the clinic to be processed. These samples were from 21.11.2018 to 21.1.2019 and accounted for 1 positive *M. bovis* CM cow from Farm 1 (which was already known), and 8 positive *M. bovis* CM cows from Farm 2.

(7) The date of detection of *M. bovis* CM was assigned as the first day of antimicrobial treatment of the case of CM that preceded the individual cow positive qPCR milk test. Positive qPCR *M. bovis* milk samples from a private commercial laboratory, Gribbles Veterinary Pathology, Palmerston North (Gribbles) were re-confirmed through AHL. For some cows, the date of QMS qPCR positive and the start date of treatment for that case, were temporally separated by days or even weeks. However, many of these cases were ATFs, with multiple courses of treatment.

3.4.4 Date of Detection of M. bovis on Study Farms

3.4.4.1 Herd-level M. bovis Status

Farms 1, 2 and 3 had different dates of *M. bovis* diagnosis. Farm 1 had a BTM qPCR positive test on 30.8.2018, and a tentative diagnosis of *M. bovis* was made. Farm 2 had a BTM qPCR positive test on 10.9.2018, with a tentative diagnosis of *M. bovis* made. Both these tentative diagnoses were confirmed by conventional PCR and sequencing at AHL. Farm 3 had a tentative diagnosis of *M. bovis* infection at a herd level made by a second round of on-farm individual cow surveillance serology (serum ELISA) being positive. The diagnosis date from serological testing was 24.10.2018.

3.4.4.2 Cow-level M. bovis Clinical Mastitis

The index cow for Farm 1. The first *M. bovis* CM case on Farm 1 was cow Tag Id.413, initially treated for CM on 15.8.2018. On the 26.9.2018, a right hind QMS tested positive on qPCR (test confirmed by AHL). On this date, the cow had received three courses of intramammary antibiotics which included

two courses of Penclox (3 syringes at 24 hour interval, Virbac NZ Ltd) and one extended course of Mastalone (6 syringes at 24 hour interval, Zoetis New Zealand Ltd). (See 3.6. Also, full details of CM antimicrobial therapies used for during this study are described in Appendix Document 1 Chapter 3, 6.1.1.) She had remained in the 'red' herd at time of testing. (The 'red' herd being cows under antibiotic treatment, within milk WHPs or 'non-cured' cows.)

The index cow for Farm 2. The first *M. bovis* CM case on Farm 2 was cow Tag Id.957, initially treated for CM on 9.9.2018. On the 18.9.2018 a milk sample for this cow tested positive on qPCR (test confirmed by AHL). On this date, the cow had received a four-day course of Tylan injection (25 ml daily, Elanco Animal Health, Auckland) for mastitis in the left front and right front quarters. She was in the 'red' herd at time of testing.

The index cow for Farm 3. The first and only *M. bovis* CM case on Farm 3, was cow Tag Id.321. Over the period 21.11.2018 to 21.1.2019 (herd depopulation dates 30.1.2019 to 10.2.2019) the farmer delivered four batches of mastitis milk samples to the clinic, from 31 mastitic cows, for processing. On the 21.1.2019, a right hind QMS from cow Tag Id.321 tested *M*. *bovis* qPCR positive. On this date, the cow had received two courses of intramammary antibiotics which included one extended course of Penclox (4 syringes at 24-hour intervals, Virbac NZ Ltd) starting 27.12.2018, and one course of Mastiplan (four syringes at 12 hour intervals, MSD Animal Health, Upper Hutt) starting 6.1.2019. She was in the 'red' herd at the time of testing.

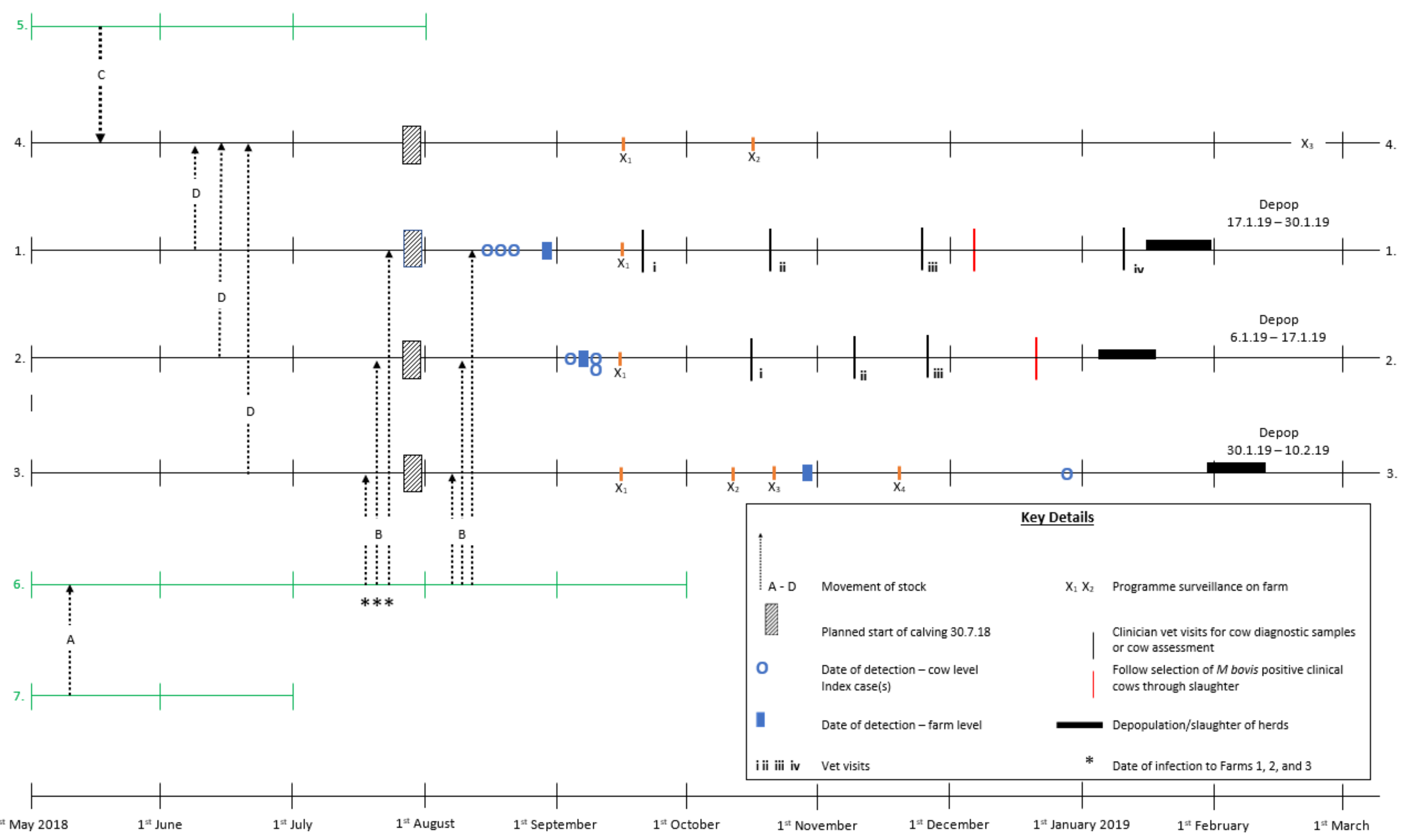


Figure 3-2 Diagrammatic representation of animal movements and sampling times in the dairy farming enterprise Including four dairy units (ID 1 – 4 on left of graphic)

3.5 Further Aspects of the Enterprise

3.5.1 Young Stock Movements

This highly integrated dairy farming operation regularly moved stock between dairy farms, run-off and grazing properties. There were 113 farmer-recorded stock transport episodes in the 17 months between 1.5.2017 and 6.9.2018.

In February/March 2017, 750 2016 spring born heifer calves, were moved from Farm 6, farmer owned run-off, to Farm 7 for grazing. The calves were from Farms 1, 2 and 3 and had been kept as separate lines (calves from each farm grazed separately). These calves returned from Farm 7 grazing, as in-calf heifers (Arrow A Fig. 3-2) to Farm 6 from 8.5.2018 to 14.5.2018. From discussion with the farm managers, there had been a variable amount of mixing with stock from other owners while on Farm 7, with Farm 1 and 2 heifers being mixed on body weight, and grazed closer to other graziers and farmer owned stock. Farm 3 heifers were grazed as a separate line and were only mixed for about 10 days with Farm 1 and 2 heifers, before returning to Farm 6. On arrival back on Farm 6, they were re-tagged and split back into their herd of origin. These heifers were treated with an internal teat sealant (Teatseal®, Zoetis NZ Ltd) for prevention of mastitis, in late June 2018. From 16.7.2018, springing heifers were returned in groups to each of Farms 1, 2 and 3. The first group saw between 60 to 90 heifers returning to their respective dairy farm, to join their springing herd. These group returns occurred approximately every 10 days (Arrows B). On 15.5.2018, the 99 in-calf heifers from Farm 4, which had been grazed on Farm 5, and never been in contact with the 750 heifers from Farm 1, 2 and 3, returned to Farm 4 (Arrow C).

MPI (personal communication, 6.9.2019) noted "*based on cattle movements, all evidence points to the 750 heifers that were introduced to three of the four (North Otago) dairy platforms in spring 2018 as the source of infection. The hypothesized infection date into the enterprise is the date these 750 heifers moved onto the enterprise in May 2018, namely the run-off. They moved onto the dairy platforms in July 2018*."

3.5.2 Cow Movement to Farm 4 in Winter 2018

In May 2018, 165 young (3 to 5-year-old), non-pregnant cows with no recorded CM during the 2017/18 season, low ISCCs (all tests for season <100,000 cell count), and with good production figures (>400 kgMS), were selected from Farms 1, 2 and 3 to be carried over and milked through on Farm 4. Seventy-two in-milk cows were moved from Farm 1 on 6.6.2018, 57 from Farm 2 on 16.6.2018 and 36 from Farm 3 on 20.6.2018. All cows were moved directly to Farm 4 from their farm of origin (Arrows D Fig. 3-2).

3.6 Antimicrobial Therapies Used for Mastitis Treatments

Both parenteral and intramammary antimicrobial therapy were used for the treatment of CM. While it is standard practice in scientific writing to use the name of the active ingredients, the trade names will be used for ease throughout this study, as several of the intramammary infusions are combination products containing up to four active ingredients. The trade name, active ingredient(s), company of manufacture, Milk Withholding Times (WHT) and Meat WHT for the eight antimicrobial therapies (Mamyzin, Tylan, Intracillin 1000 Milking Cow, Clavulox, Albiotic, Penclox, Mastiplan and Mastalone) used on Farm 1 and Farm 2 throughout the *M. bovis* CM outbreak are reported in Appendix Document 1 Chapter 3, 6.1.1. As several of the preparations have numerous registered treatment protocols, only the most commonly used treatment protocol has been included.

3.7 Methodology of Sample Collection

3.7.1 Herd-level

Bulk Tank Milk (BTM) Samples

MilkTestNZ, Hamilton (personal communication, 10.9.2020) noted that BTM samples were collected on-farm by the dairy company's tanker driver. Using single use plastic vials, two 37ml milk samples from the milk vat were collected from each farm scheduled for collection. The sample vials were delivered by the tanker to the milk processing factory. From the factory, samples were placed with ice packs in chilly bins for the courier, and transported overnight to MilkTestNZ, Hamilton for processing. Chilly bins were temperature checked on arrival to ensure the temperature has remained within required limits. Samples were processed on day of receipt, where possible.

3.7.2 Animal-level

3.7.2.1 Collection of Cow Blood Samples

A 10ml red top plastic tube (BD Vacutainer®, CAT - Clot Activator with silica) was used for serum collection. A single 18G x 1" (1.2 x 25mm) sample vacutainer needle was used for blood collection from the coccygeal vessels. On farm, the blood samples were collected by Programme staff, veterinarians, or veterinary technicians. At slaughter, post-slaughter heart blood samples were collected by Asure-Quality staff, also into a similar 10ml red top plastic tube. Blood samples were stored in vertical racks and depending on the test required, were processed at AHL or Gribbles Veterinary Pathology, Palmerston North. On collection, bloods were placed in chilly bins with icepacks, ensuring that blood samples were not in direct contact with the ice packs, and were dispatched on the day of collection to the laboratory. For sampling that crossed a weekend, samples were chilled at 4°C until dispatch.

A prospective cohort study was carried out on Farm 1. This study aimed to investigate patterns of serum ELISA test results in a herd with diagnosed *M. bovis* CM cases to estimate transmission rates over time and estimate prevalence of shedding against prevalence of serological conversion in a dairy farm, that at the time of testing was not overtly clinical.

3.7.2.2 Methodology for the On-farm Collection of Cow Palatine Tonsil Swabs for M. bovis qPCR

The methodology to swab palatine tonsils, used by the author (KPK), for CM cows on Farm 1 and 2 is detailed. Briefly, the cow was restrained in a head crush and 1.5 ml 2% Xylazine (Xylazine 2% injection, Phoenix Pharm Distributors Ltd. A5541 RVM), administered in the tail vein with a sterile 3ml syringe and 16 G x 1' (1.651mm x 2.5cm) needle. Nose grips were used for safety to restrain the cow's head. A 'gag' – 50 mm PVC (polyvinyl chloride) pipe, cut to 40 cm in length, was placed through the mouth and inserted caudally to the nasopharynx, using a head torch to provide an external light source. The palatine tonsillar crypts were visualized (Fig. 3-3). A guarded tip swab (Swab. KI-3000 Guarded Culture Instrument, 69 cm. Kalayjian Industries, Inc. California, USA) was inserted into the left and right palatine tonsillar crypts. The swab tip was rotated within the tonsil crypt to collect the samples. On withdrawing of the swab, the tip of the swab was broken off, approximately 2cm from the tip, and placed in a 10 cm red vacutainer tube. Collected samples were chilled on farm with ice-packs and placed in a chilly bin for qPCR testing at AHL Wallaceville, Wellington.

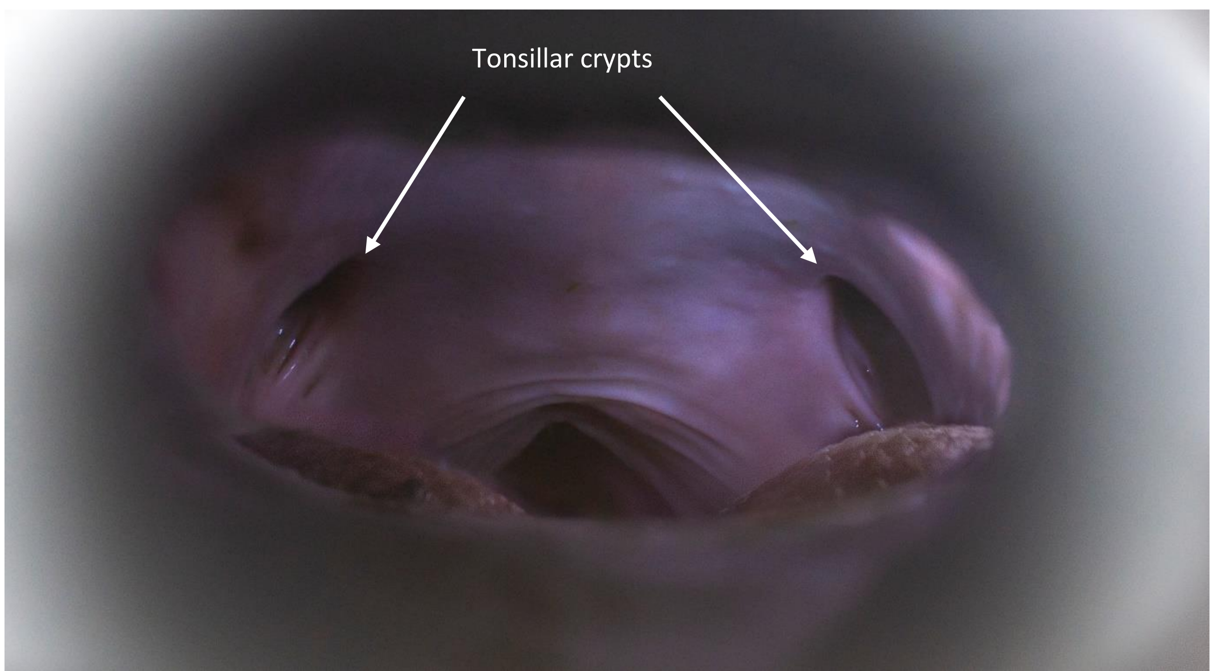


Figure 3-3 Visualization of the palatine tonsillar crypts, via PVC pipe in an adult dairy cow

3.7.2.3 *Methodology to Swab Palatine Tonsils at Slaughter*

The methodology for swabbing the palatine tonsils at slaughter was different to on-farm collection. Briefly, the detached, previously washed head was presented to the veterinarian/meat inspector for examination (Fig. 3-4). Prior to inspection, where the head inspection is described in Red Meat Code of Practice, Chapter 6 (Ministry for Primary Industries, 2015) a sterile dry 13cm swab (Cultiplast CE 005, Cod 111598, LP Italiana, Milan, Italy) was inserted into both tonsillar crypts, and vigorously rotated in order to dislodge mucosal cells and mucus. The swab was withdrawn and the entire swab placed in the swab container. (The individual carcass number was recorded on the swab container, and was to be traceable to the RFID.) Collected samples were chilled with ice-packs, placed in a chilly bin and transported to AHL Wallaceville for qPCR testing.

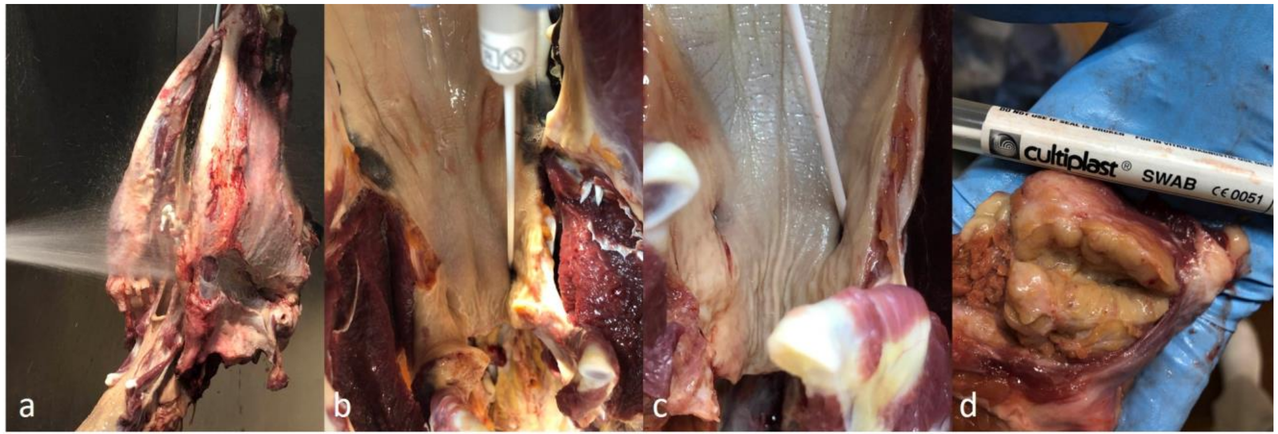


Figure 3-4 Exposed nasopharynx and palantine tonsil swab collection at slaughter
Head was high pressured hosed prior to the swab taken (a), placement of swab in tonsillar crypt (b and c), incised palatine tonsil (d).

3.7.2.4 Cow Milk Samples - Composite Milk Samples

Over the study period, individual cow CMSs were collected during routine Programme surveillance visits, and as part of the non-clinical prospective cohort study on Farm 1 (3.11). These were collected by Programme staff, veterinarians, and veterinary trained technicians.

As the individual cow mastitis samples were to be tested by qPCR and not cultured, the samples were not collected aseptically. However, the samplers wore gloves, with thorough hand washing between cows to decrease the risk of potential cross-contamination between samples. After discarding the first 2-3 strippings from a gland, the milk sample was collected into a 35 ml plastic screw cap vial (Stowers Containment Solutions, Auckland, NZ), and chilled for transport by courier. These milk samples were either processed at the AHL Wallaceville, or at Gribbles, Palmerston North.

3.7.2.5 Collection of Calf Nasal Swabs for M. bovis qPCR

A sterile dry 13cm swab (Copan 159C, Copan Diagnostics Inc, Murrieta, California, USA) was inserted up both nostrils of the calf and rubbed against the mucosal lining of the upper nostril, preventing as much contamination from the tip of the nostril as possible. Calf nasal swab samples were collected by Programme staff and stored in a 4°C fridge until couriered to AHL with ice packs, with the samples not touching the icepacks directly. On arrival at AHL, the swabs were stored at 4°C. The average time between sample collection and sample submission for processing was three days.

3.7.3 Quarter Sampling

Quarter milk samples (QMS) were also collected at the two vet visits, on Farm 1 and 2, to examine farmer-selected CM cows. Gross visual examination of expressed milk from *M. bovis* CM cows was done by the author (KPK), where possible. Methodology of collection was as described in 3.7.2.4.

In addition, between 21.11.18 and 21.1.19, the farmers from Farms 1, 2 and 3, as an aid to decision-making about therapy and culling, also delivered a number of QMS of farmer-selected CM cows to the clinic for processing. These samples, under Programme directive, were double bagged, chilled and on some occasions had been frozen. These samples were processed at Gribbles Palmerston North, for *M. bovis* qPCR analysis as well as a DNA Mastitis Pathogen Multiplex qPCR panel (3.8.1/6.1.2.7).

3.7.4 Pathology Samples of Mammary Glands from M. bovis CM Cows at Slaughter

On 4.12.2018, 39 cows from Farm 1 were sent to slaughter at Alliance Group Pukeuri Plant, Oamaru. Twenty-six of these cows had a history of *M. bovis* CM, confirmed by milk QMS qPCR. From these clinically affected cows, 14 mammary glands were randomly selected to be grossly examined and sampled for histopathology and IHC.

Gross examination of affected glands was conducted as follows: after the routine slaughter procedure, the mammary glands were detached from the carcass on the slaughter board by a meat processor and were individually labelled and identified with the carcass number, cow farm management tag, LID tag, and RFID/EID (NAIT), and then transported to an area distant from the slaughter floor for examination.

Gross examination of the individual mammary glands was conducted by the author (KPK). Visual examination of each gland consisted of sectioning individual quarters to evaluate the presence, morphology, and the distribution of gross lesions. Mammary tissue samples were collected from representative areas with obvious gross lesions, trimmed to a 1-cm thick sections, and placed in fixative solution (10% formalin) for preservation. Fixed specimens were submitted for histopathological preparation and examination at Massey University, Palmerston North. Prior to trimming for histopathology, all submitted formalin fixed mammary gland tissues were photographed. The author (KPK) then conducted visual examination of fixed photograph specimens to determine the presence of gross lesions.

3.8 Laboratory Analysis

3.8.1 DNA Extraction and M. bovis qPCR

Bulk Tank Milk, cow palatine tonsil swabs, calf nasal swabs and individual cow milk samples were processed for qPCR analysis. BMT samples were processed at MilkTestNZ, Hamilton. Palatine tonsil swabs, calf nasal swabs, and some of the individual cow milk samples were processed at AHL. The remaining individual cow milk samples were initially processed at Gribbles, Palmerston North. Gribbles milk samples which reported a qPCR detect, were further processed at AHL.

See Appendix Document 2 Chapter 3, 6.1.2 for Laboratory Methodology for:

(i) DNA extraction AHL, Wallaceville (6.1.2.1)
(ii) DNA extraction Gribbles, Palmerston North (6.1.2.2)
(iii) *Mycoplasma bovis* qPCR testing at AHL, Wallaceville (6.1.2.3)
(iv) *Mycoplasma bovis* qPCR testing at Gribbles, Palmerston North (6.1.2.4)
(v) *Mycoplasma bovis-specific* antibodies using indirect ELISA (6.1.2.5)
(vi) Histopathology and Immunohistochemistry for *M. bovis* (6.1.2.6)
(vii) DNA Mastitis Pathogen Multiplex qPCR (6.1.2.7)

3.8.2 Confirmatory Conventional PCR Testing and Sequencing at AHL

For confirmation of *M. bovis* on a property, a conventional PCR was run on a qPCR positive sample. The conventional PCR used was based on Pinnow's methodology (Pinnow et al., 2001). A 442 bp (base pairs) fragment of DNA was amplified.

With multiple qPCR positives, three samples in duplicate were run on the conventional PCR, along with a positive control, two negative controls and a ladder. Bands from the gel were cut and DNA extracted with the Zymoclean Gel DNA recovery kit. The concentration of DNA was also measured using a Qubit 3.0 Fluorometer, NanoDrop 8000 Spectrophotometer. The samples for sequencing were sent to EcoGene Sequencing Service, Landcare Research, Auckland.

EcoGene used Sanger sequencing to produce the sequencing result. The forward and reverse primer sequencings were returned to AHL, where they were assembled using the Geneious computer program. The sequence was run through a nucleotide BLAST (Basic Local Alignment Search Tool) search on NCBI-GenBank (National Centre for Biotechnology Information) in order to find a matching sequence to confirm the *M. bovis.*

3.8.3 Other Methodologies (see Appendix Document 3 Chapter 3, 6.1.3)

(i) Gribbles – Trace elements and day 3 post-calving bloods (6.1.3.1)

(ii) LIC (Livestock Improvement Corporation) BTM BVD Ag PCR and LIC BTM Ab ELISA (6.1.3.2)

(iii) Gribbles - Individual cow BVD Ag and BVD Ab (6.1.3.3)

3.9 Data Sources

3.9.1 The Programme Database

Surveillance testing results, both on-farm and at slaughter, for the NO dairy operation were sourced from the Programme database. Cows and calves tested were identified by their unique 15-digit NAIT (RFID) tag.

For analysis, these RFID numbers were linked to their alphanumeric LID (Birth ID), which included a four letter LIC participant code - year of birth - animal number. As needed the LID was further linked to Farm Management tag number.

3.9.2 The Programme Exotic Disease Investigative Report (EDIR)

An EDIR report, which was completed by an MPI veterinarian, contained detailed information about a *M. bovis* confirmed farm. The report included information sourced from the farm owner and/or manager on location of stock, stock numbers, animal and farm management, movement of animals both onto and off the farm, and details on any current or previous animal health concerns or disease investigations (Biosecurity New Zealand, 2020).

3.9.3 Infovet (Zoetis New Zealand Ltd, Auckland, NZ)

Demographic data including last recorded calving, six week in-calf rate, calving patterns, BMSCC, and body condition score (BCS) were obtained electronically from an in-clinic, computerized dairy management system, Infovet (Zoetis, Auckland).

3.9.4 MINDA®, LIC, Hamilton, NZ

Lifetime identification (LID), cow breed, age, mastitis treatments and other animal health treatment records recorded into Protrack® (Farm Automation Services, LIC) were extracted from MINDA® reports. This data was recorded in MINDA® software.

3.9.5 Vision VPT™ Covetrus Software Services, Australia

Historic and current veterinary farm records were sourced from in-clinic Vision program/database, Veterinary Centre Oamaru.

3.9.6 Farmer Records

Farm details, farmer 'red books' (animal health treatment records), stock movements, culling reports, farm management reports, and over twenty-five years of veterinary experience working with this farming operation by the author (KPK) were also used as data sources.

3.10 Statistical Analysis and Data Management

3.10.1 R Core Team (2018)

Statistical analysis was performed in R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL *https://www.R-project.org/*

The statistical significance for tests was declared at $p \leq 0.05$. Confidence intervals reported were for 95% of range of values and percentages in Tables were rounded to whole numbers.

3.10.2 Bulk Tank Milk ELISA Linear Regression

The R function *lm* (simple linear regression) was used to determine the relationship between BTM ELISA and the farms in this operation. Farm was the explanatory variable with BTM ELISA the response variable. (The temporal patterns of SP% changing over time i.e. time clustering, were ignored.)

3.10.3 Seroconversion and Seroprevalence of Farms 1 to 4

Seroconversion was measured as a serum ELISA SP value, expressed as a SP%. The within-herd seroprevalence for Farms 1 to 4 were calculated as the proportion of cows individually *M. bovis* serum ELISA sampled which had a SP% ≥60 (Parker, House, Hazelton, Bosward, Morton, et al., 2017; Petersen et al., 2016).

The Programme reported serum ELSIA results between SP% ≥60 and <80 as 'weak positive'. From 1705 individual cow serum ELISA results reported in this study, 76/1705 (4.5%) were classified as 'weak positive'. Since these 'weak positives' are over the cut point of SP% ≥60, for the purposes of this study, they have been included in test positive sample totals.

Internationally, ELISA test results have been reported as ODC% (optical density coefficient), which is similar to SP%. The SP% is calculated from optical density as (OD sample-OD negative control) / (OD positive control-OD negative control) x 100% (ID.vet, 2018).

The R function *prop.trend.test* was used to determine the trend in proportions of seroprevalence over time.

True infection prevalence was estimated using Epitools *http://epitools.ausvet.com.au* (Sergeant, 2018), using a SP% 60 cut point and serum ID.Vet ELISA individual animal test sensitivity of 89.3% and specificity of 95% (Mackereth & Marquetoux, 2019). The Epitools calculator page states "True prevalence estimates that are less than zero or greater than one are not consistent with assumed sensitivity and specificity values, and are indicated by "<0" and ">1", respectively." Where the estimate for the calculations for true prevalance for Farms 1-4, was less than zero, notably Farm 4, this has been indicated as <0, as recommended. Epitools estimated true prevalence were calculated as described by several authors (Greiner & Gardner, 2000; Reiczigel et al., 2010; Rogan & Gladen, 1978). Blaker's, Sterne, Clopper-Pearson and Wilson confidence limits are calculated as described by Reiczigel et al. (2010). The authors recommend Blaker's interval for general use of true prevalence, and this option was selected when using the Epitools calculator.

The method used to compare BTM ELISA over time and the estimated true infection prevalence was default loess smoothing using ggplot package, with stat_smooth() function. Details on the loess smoothing used: *https://rdrr.io/r/stats/loess.html*

3.10.4 Data Management for Modeling of Individual M. bovis CM Cow ELISA SP% Over Time

3.10.4.1 Date of Diagnosis of M. bovis CM

For the analysis of serum ELISA SP% of *M. bovis* CM cows, time was measured in days from the onset of the first CM case in a quarter from which there was qPCR *M. bovis* detection (3.4.3.2). For example, cow Tag Id.561, an ATF case, which received 5 courses of antibiotics in the left hind gland, was first detected with *M. bovis* by qPCR from a milk sample collected on 18.9.2018 and was seropositive with a serum ELISA of SP%=138 on the same day. The case of CM was first treated 14 days earlier on 4.9.2018, the date considered Day 0 of her *M. bovis* CM. Cow Tag Id.561 was re-blood tested on 23.11.2018 (day 80, ELISA seropositive with SP% 342), and again at slaughter on 4.12.2018 (day 91, still seropositive at SP% 114).

3.10.4.2 Generalised Additive Model for SP Values for Farm 1 and 2

The modelling of the estimated mean antibody response SP% for M. bovis CM cows has recently been carried out in a small number of cows by Petersen et al. (2018), using a generalised additive mixed model (GAMM). A similar model, a generalised additive model (GAM), has been used on the M. bovis CM cows from Farm 1 and Farm 2 in this outbreak and is reported.

The change in SP% values over time was examined with a GAM using the *gam* function from R package *mgcv* (Wood et al., 2016) with identity link to represent the gaussian family, i.e. assuming a normal distribution for the response, SP. As GAM's are non-parametric, the form of the relationship between SP% and time is not fixed, but rather, the data is used to estimate the relationship between two continuous variables as a smoothed curve. The GAM modelling of Petersen et al. (2018) included cow as a random effect, but since the variance of cow as a random effect was zero for Farm 1 data, and was not significant for Farm 2, this term was not included in the final model.

The quantile-quantile plots of the model residuals were visually assessed for failure to meet the normality assumption. The general form of the two herds were similar, but wanted to draw strength of both farms. (See Appendix Document 5 Chapter 4, 6.2.5.1)

In mathematical notation, the final model is denoted as:

$$E[y_i] \ = \ \beta_0 + \beta_1 Farm2 + f(x_i) + \epsilon_i,$$

where β_0 denotes estimated mean SP for Farm1, β_1 is the estimated difference in average SP between Farm2 and Farm1, f is a smoothing function based on thin plate regression splines, y_i, x_i and ϵ_i denote the values of SP, time and residual error respectively for the ith measurement.

Smoother line and confidence limits for the intersection of SP%=60 were extracted from the *gam* plot object.

3.10.5 Incidence of Clinical Mastitis

(i) For Farms 1 to 4, the cumulative incidence of clinical mastitis. The numerator is the total number of cows the farmer diagnosed with CM, with a recorded a mastitis treatment. The observation period is the interval between individual cow calving and depopulation for Farms 1, 2 and 3, and 31.5.2019 for Farm 4. The number of cows present at the start of the observation period for each herd, from the late September 2018 EDIR census, was used in the calculations of incidence measures.

(ii) For Farms 1 and 2, the cumulative incidence of *M. bovis* CM was calculated as the number of QMS qPCR *M. bovis* detected cows, at a cow level definition (both ATF cases and single course treatments) as the numerator, divided by the herd numbers, as above in (i).

(iii) For Farms 1 and 2, the incidence rate of first case of farmer-detected *M. bovis* CM at a cow level, was calculated by working out 'total risk days' (slaughter date – calving date +10 days for time in the springers) less 'deleted risk days' (slaughter date – date of tentative *M. bovis* CM diagnosis). Cases per cow-days were converted to cases per cow-months. The testing of a cow, once an *M. bovis* milk qPCR diagnosis was made, effectively ceased. Therefore, she was no longer considered at risk from the point of diagnosis.

3.10.6 Analysis of Age of M. bovis CM cows

Pearson's Chi-squared test, with standardised residuals, was used for this analysis. A standardised residual greater than 2 indicated a different representation compared with other groups.

The seasonal incidence, by age, of CM, was the number of heifers or cows with a CM treatment recorded in an age group, divided by the total number of heifers or cows in the age group, as from MINDA®, LIC, Hamilton.

3.10.7 Agreement Study Between Serum ELISA SP% and Tonsil Swab qPCR in Individual Cows

A study was designed to compare how well the serum ELISA (SP%) agreed with the palatine tonsil qPCR.

A number of tentatively diagnosed *M. bovis* CM cows, from a QMS, were blood tested and tonsil swabbed, on the same day, on Farm 1 and 2 and these diagnostic tests were repeated at slaughter on these same cows. On Farms 1, 2 and 3 surveillance slaughter data, again both serum ELISA and tonsil qPCR, from the same cow, on the same day were collated. This enabled agreement tests on paired samples to be carried out. Note, the sensitivity and specificity of these diagnostic tests were not being assessed, nor were the various cut points that can used when assessing tests with a continuous outcome that is catergorised into a binary 'diseased or not diseased' variable e.g. the serum ELISA.

Concordant observations between two tests on the same individuals are those which are either positive for both tests or negative for both tests (Szklo & Nieto, 2014). Agreement is the sum of concordant observations divided by the sample size. Based on the following table,

		Test 1		
		Positive	Negative	Total
Test 2	Positive	a	b	R_1
	Negative	c	d	R_2
	Total	C_1	C_2	N

$$\text{Agreement} = \frac{a+d}{a+b+c+d}$$

3.10.7.1 *Two by Two Tables in Agreement Study*

All tests will be referred to with the following 2x2 tables:

	Tonsil +ve	**Tonsil -ve**
SP +ve	a	b
SP -ve	c	d

Where:

a = Both tests call the animal positive (positive agreement)

b = Animal is ELISA positive, but tonsillar negative

c = Animal is ELISA negative, but tonsillar positive

d = Both tests call the animal negative (negative agreement)

And a+b+c+d = Total number of animals sampled (N)

3.10.7.2 *Gwet AC1*

To mitigate prevalence and bias issues seen with the Cohen's kappa (*k*) test for these data, Gwet AC1 was used to analyse this dataset (Gwet, 2008; Wongpakaran et al., 2013). Gwet AC1 inferences are the same as kappa. It gives a coefficient between -1 and 1, with a confidence interval and the Landis and Kock (1977) table of agreement can be used to interpret the value e.g. moderate agreement. A value of 1, in the denominator in Equation (1) represents perfect agreement. A value <0 is interpreted as observed agreement being worse than that expected by chance alone, i.e. there is a measure of disagreement, with -1 representing perfect disagreement.

Gwet AC1

$$AC1 = \frac{p - e(y)}{1 - e(y)} \qquad (1)$$

where,

$$p = \frac{A + D}{N}$$

and,

e(γ)= the chance agreement probability = 2q (1-q),

$$q = \frac{A1 + B1}{2N}$$

and A, D, A1 and B1 are defined in Table 2 (taken from Wongpakaran 2013)

Table 2 Distribution of subjects - by rater and response category

	Rater 1		
Rater 2	Category 1	Category 2	Total
Category 1	A	B	B1 (A+B)
Category 2	C	D	B2 (C+D)
	A1 (A+C)	A2 (B+D)	N

Analysis was carried out using the 'irrCAC' package in R, and the gwet.ac1.table function *https://cran.r-project.org/web/packages/irrCAC/index.html*

3.10.7.3 Landis and Kock Table of Interpretation of the Kappa Statistic of Agreement

There are different classification systems for the κ statistic, the most common being that from Landis and Kock (1977)(Dohoo, 2014);

0	agreement equivalent to chance
0.01 - 0.2	slight agreement
0.21 - 0.4	fair agreement
0.41 - 0.6	moderate agreement
0.61 - 0.8	substantial agreement
0.81 - 0.99	near perfect agreement
1	perfect agreement

3.11 Prospective Cohort Study on Farm 1

A prospective cohort study was carried out on Farm 1 during this outbreak, with the author (KPK) collecting on-farm samples of serum ELISAs and individual cow CMS on 10.1.2019. While a full description of this study, where the rationale is briefly outlined in 3.7.2.1, is outside the main objectives of this thesis, some data from this study will be used in Chapter 4 Results. This data allowed investigation of (i) seroconversion of non-clinical cows in an infected herd and (ii) prevalence and change of prevalence of SCM IMI (if *M. bovis* detected on milk qPCR)

For further detail on the methodology of the prospective cohort study see Appendix Document 4 Chapter 3.

3.12 Ethics approval

(i) On-farm *M. bovis* diagnostic sampling was carried out under Section 121 of the NZ Biosecurity Act 1993 (Government, 1993) for diagnosis of an Unwanted Organism. Samples were collected under the direction of an Authorised Person under the Act.

(ii) Massey University Human Ethics Approval SOA 19/60.

(iii) Sample collection at slaughter was carried out under Section 122 of the NZ Biosecurity Act 1993 (Government, 1993), where slaughter was part of the biosecurity response for culling for disease control. Samples were collected under the direction of an Authorised Person under the Act.

Chapter 4 Results

Overview

The Results of this case study are presented in **3 Parts**, where Part 1 reports the Outbreak Investigation (Objective 1), Part 2 the Agreement Study (Objective 2) and Part 3 the Pathological Findings (Objective 3).

Part 1 reports the main epidemiological and clinical findings in the Outbreak Investigation and is divided into three sections.

<u>4.1 Herd-level results</u> consider the *M. bovis* Programme surveillance herd-level outcomes including BTM PCR (4.1.1), BTM ELISA (4.1.2), distribution of SP% over time for the four farms aggregated at sample level (4.1.4), estimated true infection seroprevalence (4.1.5) and a comparison over time between BTM ELISA and true prevalence (4.1.6). Farm data on herd level BMSCC for study Farms 1-4 is reported as Appendix Document 1 Chapter 4, 6.2.1.

<u>4.2 Animal-level results</u> describe individual cow-level findings and begins with an overview of both the important cow-level surveillance and diagnostic tests performed and results (4.2.1 and 4.2.2). The serum SP% of individual *M. bovis* CM mastitis cows are presented, together with a model of serum SP% over time (4.2.3). Incidence of mastitis (4.2.4), age of CM cows (4.2.5) and subclinical IMI from CMS sampling (4.2.6) are also reported. Calf nasal swab surveillance testing results, on Farms 1-4, are reported (4.2.7).

<u>4.3 Quarter-level results</u> present quarter-level findings for *M. bovis* CM cows on study Farms 1 and 2. These include the presence of other pathogens in QMS samples with *M. bovis* (4.3.2) and clinical findings on the presentation of *M. bovis* CM during the outbreak (4.3.3), including antimicrobial therapy used (4.3.3.6).

In a small number of data sets there is cross over between cow-level findings and quarter-level findings and they will be reported in the appropriate section.

Part 2 (4.4) reports results of an Agreement study between serum ELISA and palatine tonsil swab qPCR test results in a sample of cows with *M. bovis* CM and another group of non-clinical cows that had surveillance samples collected at slaughter.

Part 3 (4.5) reports Pathological Findings and a description of the gross lesions seen at slaughter in *M. bovis* CM cows where the findings from 55 quarters (14 cows) are presented. Common morphological lesions, characterization of lesions (by histopathology), special stains used and IHC outcomes are outlined.

Part 1: Outbreak Investigation: Epidemiological and Clinical Findings

4.1 Herd-level Results

4.1.1 Bulk Tank Milk PCR

From 30.8.2018 when the first Programme BTM qPCR surveillance samples were collected on this farming operation, and Farm 1 returned a qPCR detection which was subsequently confirmed *M. bovis* positive by sequencing, until 4.3.2019 regular BTM qPCR (and BTM ELISA) testing was performed. Both Farms 1 and 2 provided BTM qPCR samples with varying proportions of 'detected' results throughout the testing period, but 'no detect' results were reported from Farms 3 and 4 (Table 4-1 and Fig. 4-1).

Table 4-1 Bulk tank milk (BTM) Mycoplasma bovis qPCR test results from study Farms 1 – 4 sampled between 30.8.2018 and 4.3.2019

Farm	Nos. of samples	qPCR detected [1]	% qPCR detected
Farm 1	52	43	83
Farm 2	50	20	40
Farm 3	54	0	0
Farm 4	85	0	0

[1] The Programme defined a BTM qPCR detection when *M. bovis* was detected at a cycle threshold (CT) < 45 cycles.

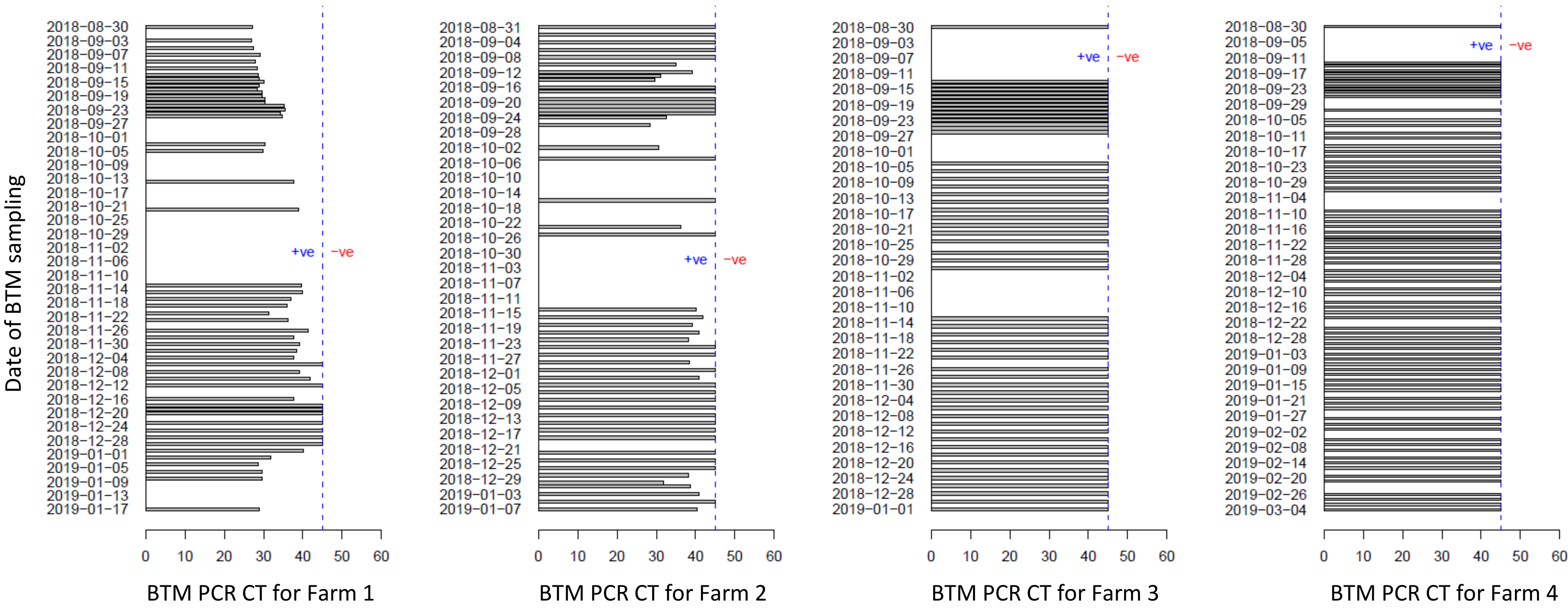


Figure 4-1 Bulk tank milk (BTM)[1] Mycoplasma bovis PCR test results across 4 study farms sampled between 30.8.2018 and 4.3.2019

[1] BTM PCR CT (cycle time) values were not reported above 45, when declared negative.

4.1.2 Bulk Tank Milk ELISA Across the Four Study Farms

Farms 1, 2 and 3 provided BTM ELISA samples with varying proportions of positive results throughout the testing period, but no positive results were reported from Farm 4 (Table 4-2 and Fig. 4-2).

Table 4-2 Bulk tank milk (BTM) Mycoplasma bovis ELISA test results from study Farms 1 – 4 sampled 30.8.2018 and 4.3.2019

Farm	Nos. samples	ELISA positive	% ELISA positive
Farm 1	33	31	94
Farm 2	30	29	97
Farm 3	31	22	71
Farm 4	4	0	0

The first positive BTM ELISA for Farm 3 was later (24.10.2018) than Farms 1 and 2, and the SP% following that date were less than those of Farms 1 and 2.

For Farm 1, there were 23 simultaneously positive days, where both BTM qPCR and BTM ELISA were positive and 13 for Farm 2.

4.1.3 Bulk Tank Milk ELISA Linear Regression

Linear regression results across the entire collection period from late August 2018 to Jan 2019, ignoring the temporal correlation between samples from the same farm, show the BTM ELISA SP% was similar for Farm 1 and Farm 2, as the confidence intervals went from -7 to +4, a small range, and overlapped 0 for Farm 2. However, the mean SP% on Farm 3 was 16.9 units less ($p<0.0001$) than Farm 1.

Table 4-3 Regression output of bulk tank milk (BTM) ELISA and Farm

Variable	Coefficient	Std Error	Lower 95% CI	Upper 95% CI	p value
Intercept	51.58	1.93			
Farm 1	*Reference*				<0.0001
Farm 2	-1.75	2.80	-7.24	3.74	
Farm 3	-16.89	2.78	-22.33	-11.44	
Farm 4	-44.42	5.88	-55.95	-32.89	

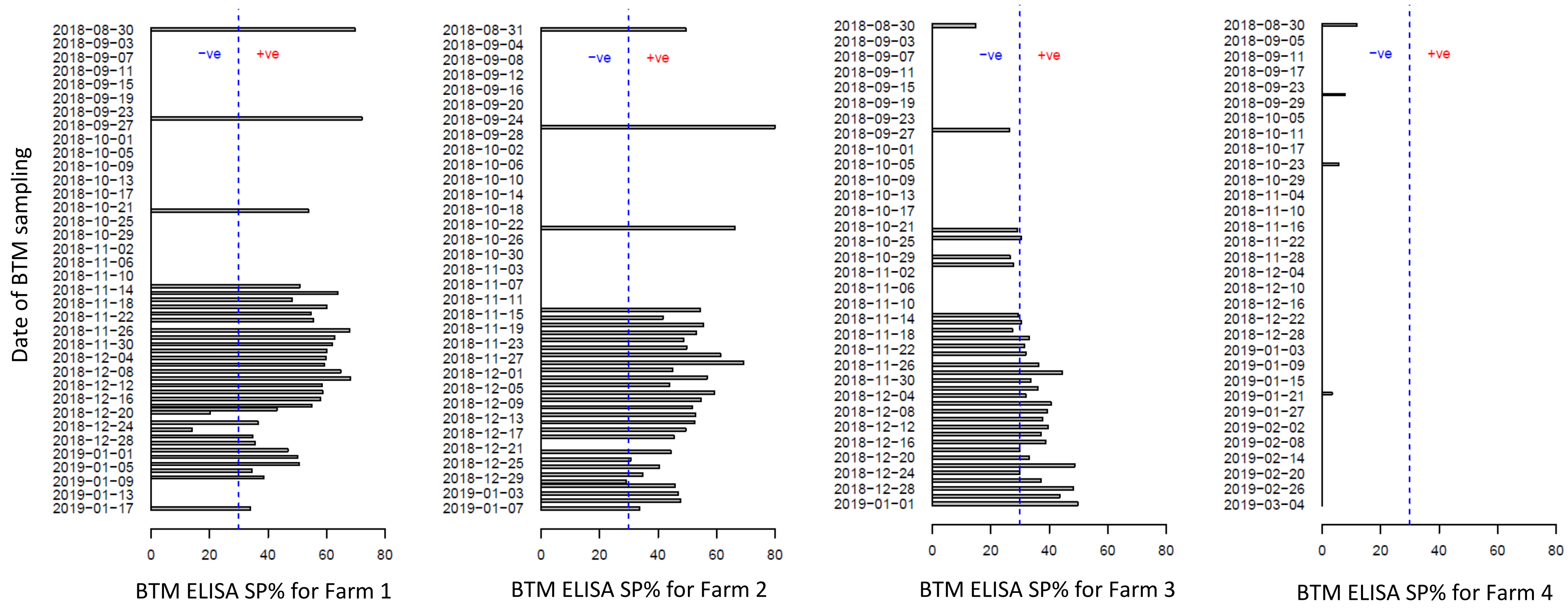


Figure 4-2 Bulk tank milk (BTM)[1] Mycoplasma bovis ELISA across 4 study farms sampled between 30.8.2018 and 4.3.2019

[1] BTM ELISA SP% declared positive > 30

4.1.4 *Distribution of SP% Over Time for Study Farms 1-4 Aggregated by Farm Sampling Date*

Individual cow serum ELISA's were collected both as part of Programme surveillance on-farm and at slaughter, and this raw data is aggregated by farm sampling date in the box plots in Fig. 4-3. The results for three Programme surveillance slaughter events, for Farm 2, have been combined into a single boxplot for readability, as the three slaughter events took place over a four-day period. For estimated true infection prevalence (4.1.5) the three data sets have been separated, to enable more detail.

The dates and number tested on each farm are presented in Table 4-4. On-farm surveillance usually saw 100 random cows blood tested, but on a small number of testing dates, specific cows were targeted i.e. Farm 1, Visit 2 cows blood tested were part of a prospective cohort study and Farm 3, Visit 4 where seroconversion in two year old heifers was being looked at. Slaughter surveillance numbers varied due to number of cows trucked on any given day, number of cows in a slaughter line, and the logistics of getting cows blood tested at slaughter. Table 4-5 reports the age of cows at each surveillance Visit 1.

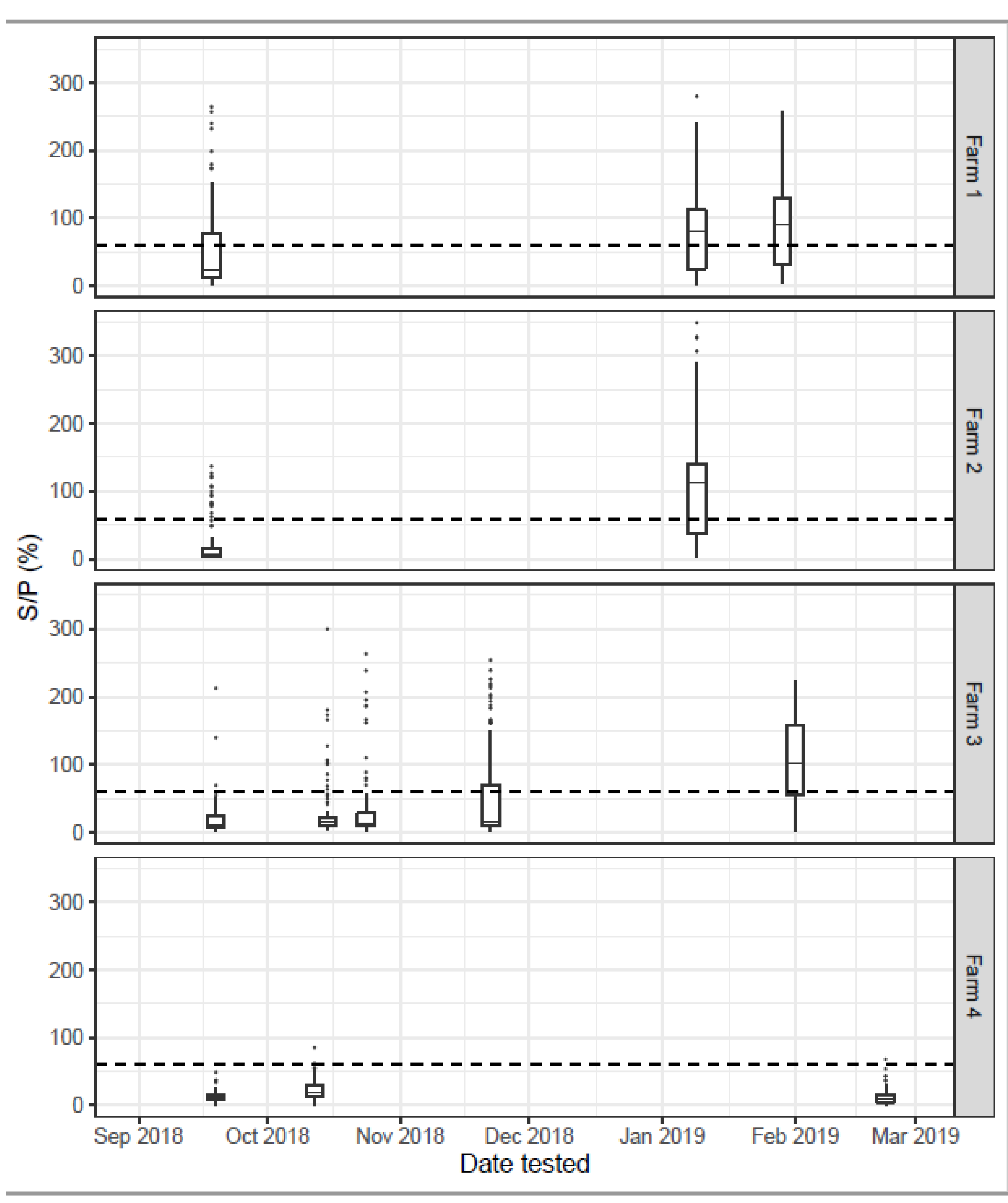


Figure 4-3 Distribution of individual cow M. bovis SP% for Farms 1-4 aggregated from individual up to herd-level, by farm sampling date

Black dotted line is cut-point for positive test results for SP% ≥60

Table 4-4 Count and proportion of individual cow Mycoplasma bovis serum ELISA test positive results for on farm surveillance visits and slaughter surveillance for study Farms 1-4

	Farm 1		Farm 2		Farm 3		Farm 4	
Visit	Sample date	Proportion test positive (%)	Sample date	Proportion test positive (%)	Sample date	Proportion test positive (%)	Sample date	Proportion test positive (%)
1	18.9.2018	33/100 (33)	18.9.2018	16/100 (16)	19.9.2018	3/100 (3)	19.9.2018	0/100 (0)
2	10.1.2019	86/133 (65)	7.1.2019	52/83 (63)	15.10.2018	14/100 (14)	12.10.2018	3/90 (3)
3	29.1.2019	55/82 (67)	10.1.2019	40/54 (74)	24.10.2018	13/100 (13)	22.2.2019	1/100 (1)
4			11.1.2019	72/93 (77)	22.11.2018	55/200 (28)		
5					1.2.2019	53/77 (69)		

Key

Farms 1-3 provided individual cow *M. bovis* ELISA samples with varying proportions of positive results at surveillance visit 1, but no positive results were reported from Farm 4 (Table 4-4 and 4-5). Farms 1, 2 and 3 had two-year old heifers which had seroconverted at the first surveillance visit, with Farm 1 being the highest proportionally.

Table 4-5 Age group prevalence of Mycoplasma bovis serum ELISA test results for on farm surveillance Visit 1 for study Farms 1-4.

	Age	2	3-5	6-9+	
Farm	Date	Proportion test positive (%)	Proportion test positive (%)	Proportion test positive (%)	**Total [1] Proportion test positive (%)**
1	18.9.2018	6/13 (46)	16/53 (30)	11/29 (38)	**33/95 (35)**
2	19.9.2018	4/16 (25)	8/61 (13)	3/18 (17)	**15/95 (16)**
3	18.9.2018	2/22 (9)	0/29 (0)	1/48 (2)	**3/99 (3)**
4	19.9.2018	0/12 (0)	0/57 (0)	0/18 (0)	**0/87 (0)**
Total		**12/63 (19)**	**24/200 (12)**	**15/113 (13)**	**51/376 (14)**

[1] A small number of cows on Farms 1, 2 and 3, and 13 Farm 4 cows, from the 100 cows blood tested at surveillance Visit 1, had missing age information.

Background Information on Farm 4, Visit 1

On Farm 4, at least 34 of the 100 cows blood tested as part of the Programme surveillance Visit 1 on 19.9.2018 had originated from Farms 1, 2 and 3 and had been moved to Farm 4, between 6.6.2018 and 20.6.2018, outlined in 3.5.2. Not all the cows moved to Farm 4 could be back traced with complete accuracy to their farm of origin. This group of 34 cows was made of 18 cows from Farm 1 (ten 3-year olds, five 4-year olds and three 5-year olds); 10 cows from Farm 2 (four 3-year olds, three 4-year olds and three 5-year olds), and 6 cows from Farm 3 (one 3-year old, four 4-year olds, one 5-year old). At the second Programme surveillance visit to Farm 4, on 12.10.2018, at least 22 of the 90 cows that were blood tested had also originated from Farms 1, 2 and 3 (Appendix Document 2 Chapter 4, Table 6-2). All cows which originated from these farms tested serum ELISA negative.

4.1.5 Estimated True Infection Prevalence

The true infection prevalence for each of these surveillance visits was estimated using Epitools (Sergeant, 2018) with results presented in Fig. 4-4 (and Appendix Document 3 Chap 4, Table 6-3).

Descriptive Statistics of True Prevalence

At the first surveillance visit, either 18.9.2018 or 19.9.2018, the true prevalence of the farms varied. Farm 1 was 0.33 (95%CI: 0.23-0.45), Farm 2 was 0.13 (95%CI: 0.06-0.23), Farm 3 was <0 (95%CI: <0-0.04) and Farm 4 was <0 (95%CI: <0-<0). By early Jan/Feb 2019 at the last sampling for Farms 1, 2 and 3, the true prevalence on these farms was similar. Farm 1 was 0.74 (95%CI: 0.61-0.85), Farm 2 was 0.86 (95%CI: 0.75-0.95), Farm 3 was 0.76 (95%CI: 0.63-0.87) and Farm 4 did not change at <0 (95%CI: <0-0.01).

Trends in True Prevalence

There was a significant trend of increasing estimated true prevalence for Farms 1, 2 and 3 over time from a trend test. There was no evidence of a trend over time in Farm 4.

Table 4-6 Trends in true prevalence for study Farms 1-4

	Farm 1	**Farm 2**	**Farm 3**	**Farm 4**
X-squared (df = 1)	21.291	74.075	87.803	0.3257
p-value	3.946e-06	< 2.2e-16	<2.2e-16	0.5682

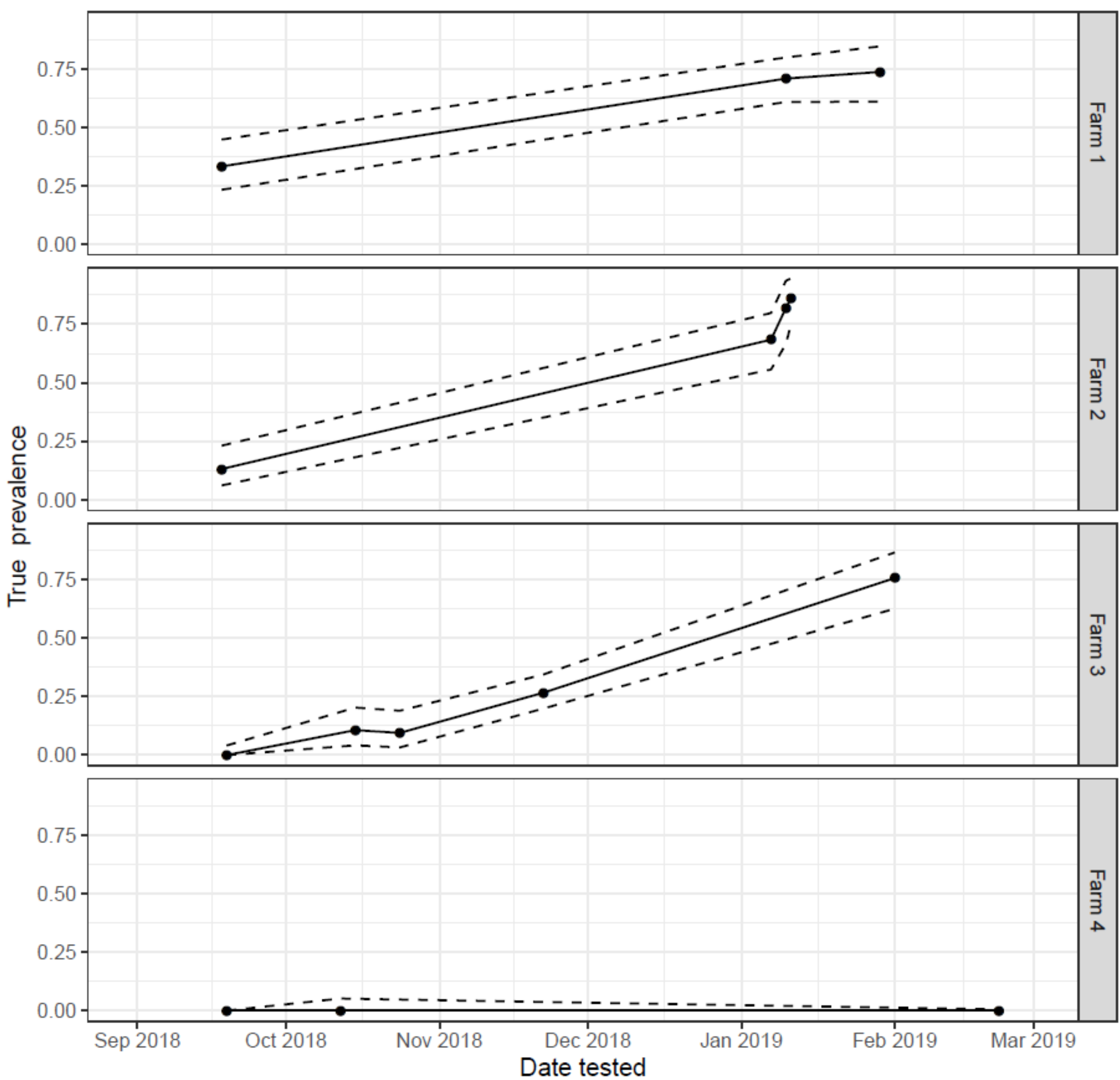


Figure 4-4 True Prevalence for study Farms 1 – 4 for Serum ELISA surveillance testing of cows

4.1.6 Comparison Over Time of BTM ELISA and Estimated True Infection Prevalence

The BTM ELISA results for the surveillance visits Farms 1-4 have been plotted against the true infection prevalence of the four farms over the outbreak time period, Fig. 4-5.

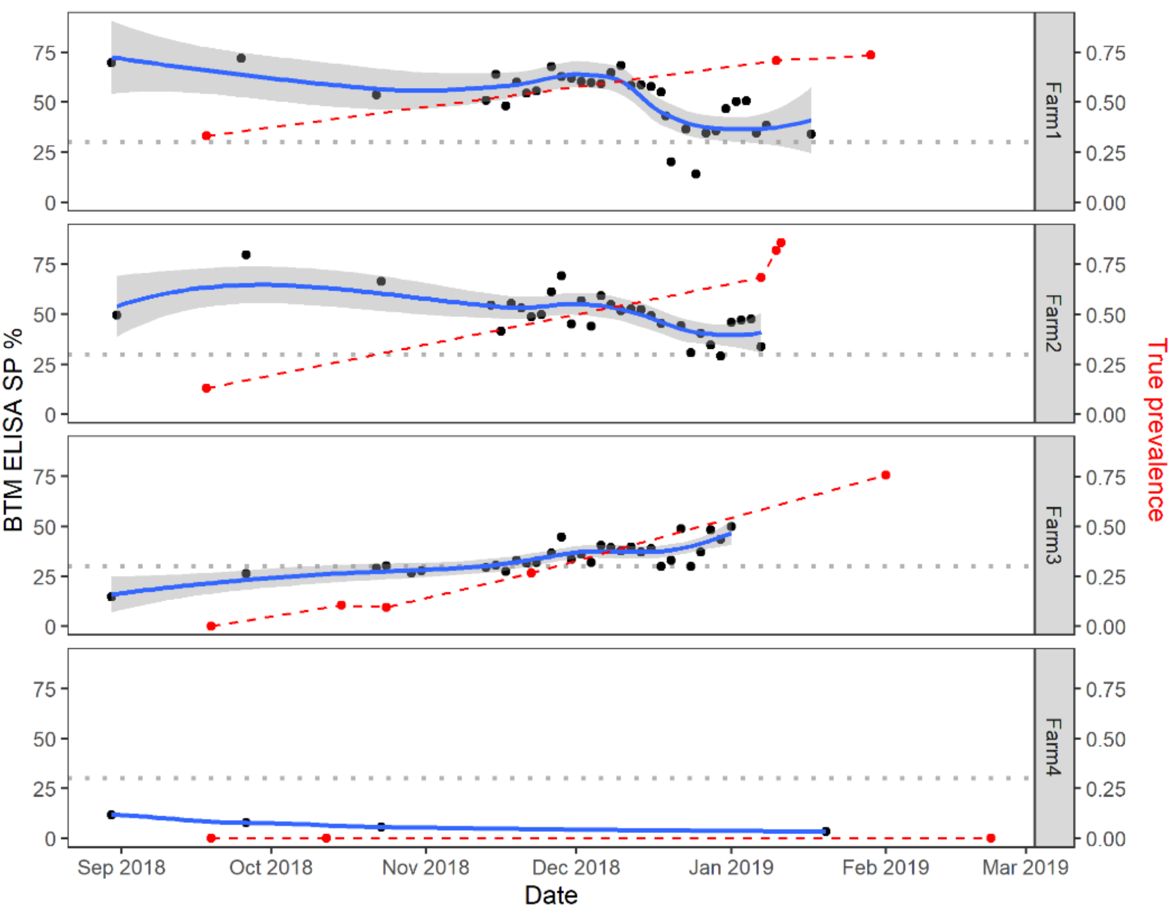


Figure 4-5 Bulk tank milk ELISA (black dots) and estimated true infection prevalence (red dots) from serum sampling over time for study Farms 1-4

Blue line is BTM ELISA, with 95% CI's as grey shading, true infection prevalence is red dotted line, black-dotted horizontal line is cut-point for positive test result

On Farms 1 and 2, the BTM ELISA decreased from mid-December 2018, while the true prevalence of infection, measured by individual cow serum ELISA increased. Farm 3 BTM ELISA and true prevalence appeared to increase with time. Farm 4 remained negative for both measures and ran parallel to each other.

4.2 Animal-level Results

This section reports the *M. bovis* CM outbreak, at an individual cow-level, and data have been sourced from different types of visits, described in Table 3-2. Visits and sample collection included Programme surveillance visits of individual cow CMS; vet visits, by author (KPK), for collection of QMS, blood tests and palatine tonsil swabs samples from farmer-selected CM cows from Farms 1 and 2; and QMS delivered to the clinic, by Farms 1, 2 and 3, under Programme instructions.

4.2.1 Individual Cow Composite Milk Sampled (CMS) at Programme Surveillance Visits

Around 25 cows had an individual CMS taken, for qPCR at Programme surveillance Visit 1 for Farms 1 – 4, and then subsequent Programme surveillance visits for Farms 3 and 4. These cows were selected by Programme personnel at the visit and comprised part of the group of cows that were also serum ELISA tested. See Table 4-7.

Table 4-7 Individual cow milk samples for qPCR at Programme surveillance visits for study Farms 1-4, proportion test detected.

	Visit 1	Proportion qPCR Detected (%)	Visit 2	Proportion qPCR Detected (%)	Visit 3	Proportion qPCR Detected (%)
Farm 1	18.9.2018	16/26 (62)				
Farm 2	18.9.2018	2/25 (8)				
Farm 3	19.9.2018	0/25 (0)	15.10.2018	0/25 (0)	22.11.2018	0/32 (0)
Farm 4	19.9.2018	0/25 (0)	12.10.2018	0/26 (0)	22.2.2019	0/25 (0)

Programme Surveillance Visit 1 cows were sampled on the 18.9.2018 or 19.9.2018 and provide important findings, especially for Farms 1 and 2, as they are the first individual cow samples collected. Infovet, MINDA® and mastitis treatment records were used to gather background information on these cows, especially calving date and mastitis treatment records to establish if they were likely to be in the colostrum mob (less than four or five days calved), and CM treatment history. Appendix Document 4 Chapter 4, Table 6-4 provides historic details, where possible, on these cows.

For Farm 1, of the 16 cows with *M. bovis* positive qPCR milk samples, 13 were under treatment for CM or milk WHP, and one cow had insufficient information about her current treatment, but had been previously treated for CM. At least nine cows were in the colostrum mob, by calving date, with one

cow not having a calving date entered. Two cows were considered as having a subclinical IMI, as no mastitis treatment had been recorded. Thirteen of the milk qPCR positive cows were serum ELISA positive.

For Farm 2, of the two cows with *M. bovis* positive qPCR milk samples, one cow was under treatment for CM, and the other in the colostrum mob, less than 48 hours calved. Neither of these cows was serum ELISA positive.

4.2.2 Vet Visits to Study Farms 1 and 2 for Collection of Samples from Farmer-selected CM Cows

A range of samples were collected from CM cows. These included QMS for *M. bovis* qPCR, palatine tonsil swabs for *M. bovis* qPCR, serum blood test for *M. bovis* ELISA and serum blood test for both BVDV Ag and BVD Ab.

Table 4-8 Count and proportion results for sampling carried out at vet visits of farmer-selected clinical mastitis (CM) cows on study Farms 1 and 2

			Proportion test detected QMS *M. bovis* PCR (%)	**Proportion test detected tonsil *M. bovis* PCR (%)**	**Proportion test positive Serum ELISA *M. bovis* (%)**	**Proportion test positive BVDV Ag ELISA (%)**	**Proportion test positive BVDV Ab ELISA (%)**
Farm 1	Visit 1	**26.9.2018**	15/16 (94)	15/15 (100)			
	Visit 4	**23.11.2018**	43/43 (100)	37/43 (86)	40/44 (90)	0/44 (0)	34/44 (77)
Farm 2	Visit 1	**17.10.2018**	22/27 (82)	26/26 (100)	17/27 (63)		
	Visit 3	**28.11.2018**	31/36 (86)	36/36 (100)	33/36 (92)	0/36 (0)	24/36 (67)

A high proportion of the CM cows presented by the farmers, on both farms, were QMS qPCR detected, palatine tonsil qPCR detected, and a variable proportion were serum ELISA positive. No cows were BVDV Ag positive i.e. PI cows for BVDV.

4.2.3 Mycoplasma bovis CM Individual Cow SP% Over Time

The serum ELISA results for cows diagnosed with *M. bovis* CM have been plotted over time. These results are from a number of testing visits including surveillance testing, both on-farm and at slaughter as described in 4.1.4, and clinical visits, in 4.2.2. Forty-four *M. bovis* CM cows had serum ELISA results included and 39 cows from Farm 2. The asterisks in the figures below represent cows with only one blood test result.

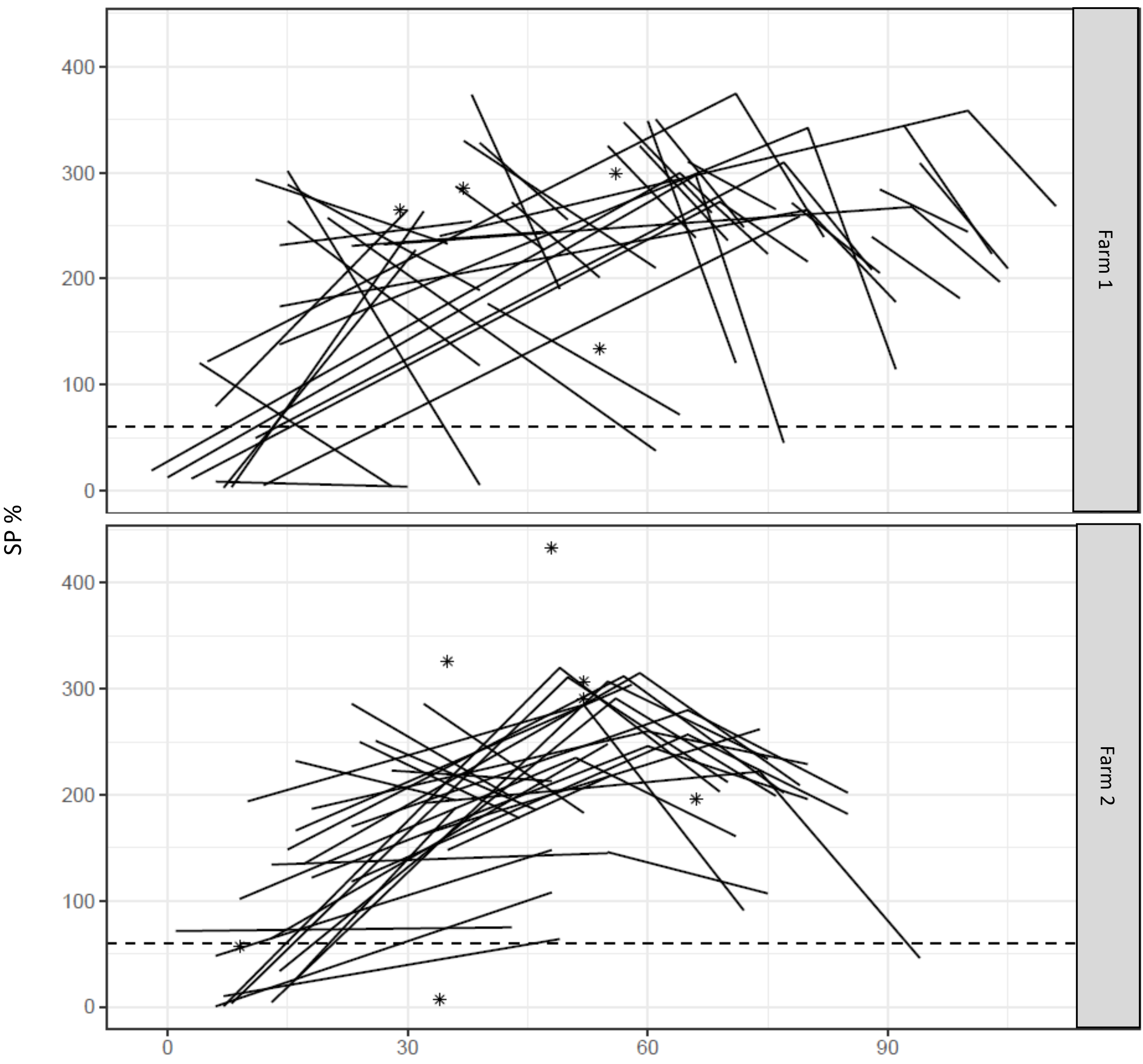


Figure 4-6 Farm 1 and Farm 2 plot of individual cows SP% over time

4.2.3.1 *Normality Plots*

The normality plots of residuals for Farm 1 and Farm 2, met the normality assumption and are presented in Appendix Document 5 Chap 4 (6.2.5.1), as are the results for the model with SP% as response for Farm 1 and Farm 2 (6.2.5.2).

4.2.3.2 *Results for the Model with SP% as Response for Farm 1 and Farm 2 Combined Data*

Table 4-9 Results of the final general additive model of the association of SP% with estimated time since diagnosis for combined data for Farm 1 and Farm 2

1. Parametric Component

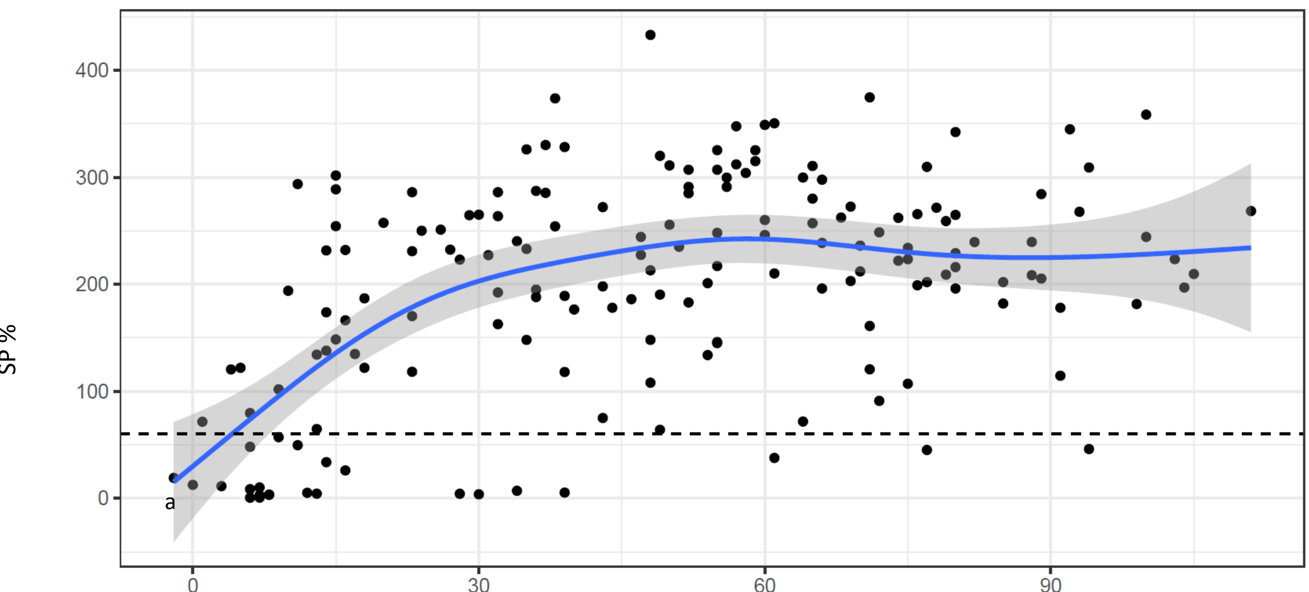
	Mean SP% (Estimate)	**Std Error**	**t value**	**Pr (>|t|)**
(Intercept)	214.39	8.62	24.871	<2e-16 ***
Farm 2	-33.57	12.73	-2.637	0.00916 **

2. Smooth (non-parametric) component

	edf	**Ref. df**	**F-value**	**p-value**
s (Time)	4.21	5.21	17.41	<2e-16 ***

The model of combined data indicated the mean SP% for Farm 1 is 214.39 and the estimate for Farm 2 is 180.82 (214.39 – 33.57). The mean SP% value across Farm 2 is significantly less than the mean SP% for Farm 1, by the value of approximately 33. The complexity of the smoothing curve, with an estimated degrees of freedom (edf) of 4.21 is slightly more than a quadratic polynomial.

4.2.3.3 SP% with Smoother Function for Farm 1 and Farm 2 Combined Data



Time (days from first diagnosis of a case of clinical mastitis from which *M. bovis* was also detected)

Figure 4-7 Smoothed relationship between M. bovis serum ELISA SP% and time from first diagnosis of clinical mastitis from which M. bovis was also detected from a general additive model of data from Farms 1 and 2

Black dotted line is cut point for positive test results for SP% ≥ 60

[a] cow Tag Id. 34 (KYDV-14-146) was sampled on 18.9.18 as Programme surveillance cow. She was not diagnosed with CM on that day, was milk qPCR positive, but serum negative (ELISA SP% = 19). The farmer diagnosed CM in the LH quarter 2 days later on 20.9.18 and she became an ATF CM case. No calving date was available, but she was in colostrum mob, and therefore assumed less than four days calved. The same cow was repeat QMS qPCR positive (LH) on-farm on 23.11.2018 and had an ELISA SP% of 299.7. At slaughter (4.12.2018) she was cow #4 for mammary gland examination – the LH had a granulomatous inflammation and all four quarters were positive for an IHC test for *M. bovis*.

The smoothed function for the combined data, cuts the line SP% = 60, at Day 5 from qPCR milk positive test. The diagnosis of CM and a cow being serum ELISA test positive was 5 days but could have been as few as -2 days and as many as 7 days, following the initial diagnosis. All infected cows are likely to be seropositive 7 days (lower 95% CI intersects SP%=60) post diagnosis, but a small number of *M. bovis* CM cows did not seroconvert at all during the observation period. The maximum value for the smoothed curve (mean SP%) for the farms combined is SP% = 262.8 and was reached at day 57 from the CM diagnosis and remained relatively constant until approximately 100 days when observations stopped.

4.2.4 Incidence of Clinical Mastitis

The incidence of CM has been considered at a herd level firstly, as cumulative incidence of CM and secondly, the cumulative incidence of *M. bovis* CM. At a cow level, the incidence rate of first cases of farmer-detected *M. bovis* CM per 100-cow-months was also determined.

Table 4-10 Cumulative Incidence (herd level) and Incidence rate (cow level) of clinical mastitis on study Farms 1-4

	Number of cows in herd	**Number of CM cases**	**Number of *M bovis* CM cases**	**Number of cow - months**	**Cumulative incidence of CM**	**Cumulative incidence of *M. bovis* CM[1]**	**Incidence rate of first cases of *M. bovis* CM per 100-cow-months**
Farm 1	1050	146	48	5066	0.139	0.046	0.95
Farm 2	1000	132	42	4826	0.132	0.042	0.87
Farm 3	705	122			0.173		
Farm 4	630	76			0.12		

[1] All cases of CM were not QMS for *M. bovis* qPCR

On Farms 1 and 2, which experienced an outbreak of *M. bovis* CM, the three measures of incidence were similar. However, in this observational study, not all CM cases were milk sampled and tested by *M. bovis* qPCR, as shown by the stacked bar plots in Fig. 4-8 and Fig. 4-9. Cows were detected with *M. bovis* CM earlier on Farm 1 than Farm 2. This matches the earlier herd level diagnosis of *M. bovis* on Farm 1 compared with Farm 2 farms by BTM qPCR testing, 15.8.2018 and 9.9.2018 respectively.

The stacked bar plots below show the cases of recorded CM per week for Farms 1 and 2 throughout the outbreak.

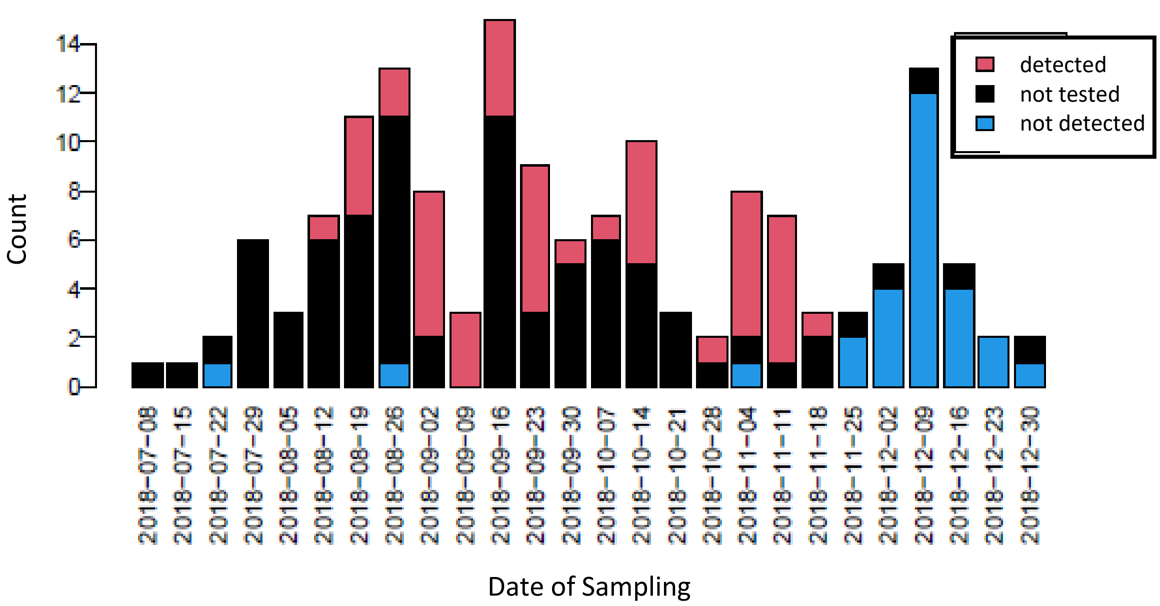


Figure 4-8 Farm 1 Stacked bar plot of CM cases per week at cow level, and outcome of individual cow milk M. bovis qPCR testing for cases sampled

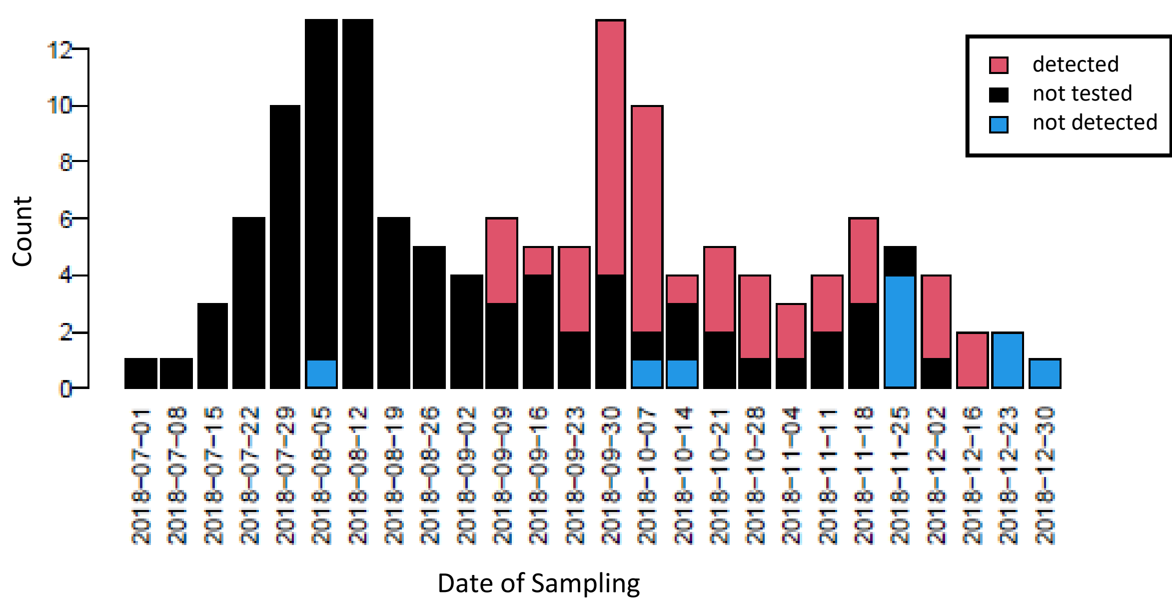


Figure 4-9 Farm 2 Stacked bar plot of CM cases per week at cow level, and outcome of individual cow milk M. bovis qPCR testing for cases sampled

4.2.5 Age of M. bovis CM Cows and CM Cows for Farms 1 and 2

A Pearson's Chi-Squared test was performed on the *M. bovis* CM incidence per age group from 2-year-olds to 9+-year-old for Farm 1 (Table 4-11) and Farm 2 (Table 4-12). As the p-values were <0.05 for both farms, suggesting that not all age groups were equally represented in disease incidence, the standardised residuals of the χ^2 test were used to indicate which age groups were over or under-represented, Tables 4-13 and 4-14.

Table 4-11 Counts of disease incidence for Farm 1

Age	2	3	4	5	6	7	8	9+
Diseased	4	6	11	7	4	9	4	3
Not diseased	257	238	181	102	73	80	49	120

Results of χ^2 test for Farm 1

Statistic	P value
19.33	0.0060

Table 4-12 Counts of disease incidence for Farm 2

Age	2	3	4	5	6	7	8	9+
Diseased	5	3	8	10	4	1	8	3
Not diseased	254	156	211	227	13	30	134	31

Results of χ^2 test for Farm 2

Statistic	P value
25.81	0.0030

Table 4-13 Standardised residuals for Farm 1

Age	2	3	4	5	6	7	8	9+
Diseased	-2.43	-1.51	1.17	1.23	0.46	2.91	1.25	-1.02
Not diseased	2.43	1.51	-1.17	-1.23	-0.46	-2.91	-1.25	1.02

Table 4-14 Standardised residuals for Farm 2

Age	2	3	4	5	6	7	8	9+
Diseased	-1.82	-1.38	-0.15	0.36	4.27	-0.18	1.20	1.54
Not diseased	1.82	1.38	0.15	-0.36	-4.27	0.18	-1.20	-1.54

Considering a standardised residual greater than 2 indicates a different representation compared with other groups, for Farm 1, cows aged 2 years are under-represented in disease incidence on Farm 1, while cows aged 7 are over-represented. On Farm 2, many more cows than expected of age 6 were diseased, the associated standardised residual is 4.27, much larger than the cut-off value of 2.0.

4.2.5.1 *Seasonal Incidence of Clinical Mastitis by Age*

The seasonal incidence, by age, for recorded CM cases, *M. bovis* CM cases for Farm 1 and 2 are plotted in Fig. 4-10, beside the national data for CM in the 2018 season, for all LIC herds which recorded CM cases. This limited data set, without confidence intervals, would suggest that in these herds, the incidence of CM on Farms 1 and 2 was greater than the national incidence, except for the heifers on Farm 1 and much greater in 6-year olds and older on both farms, compared to the national incidence.

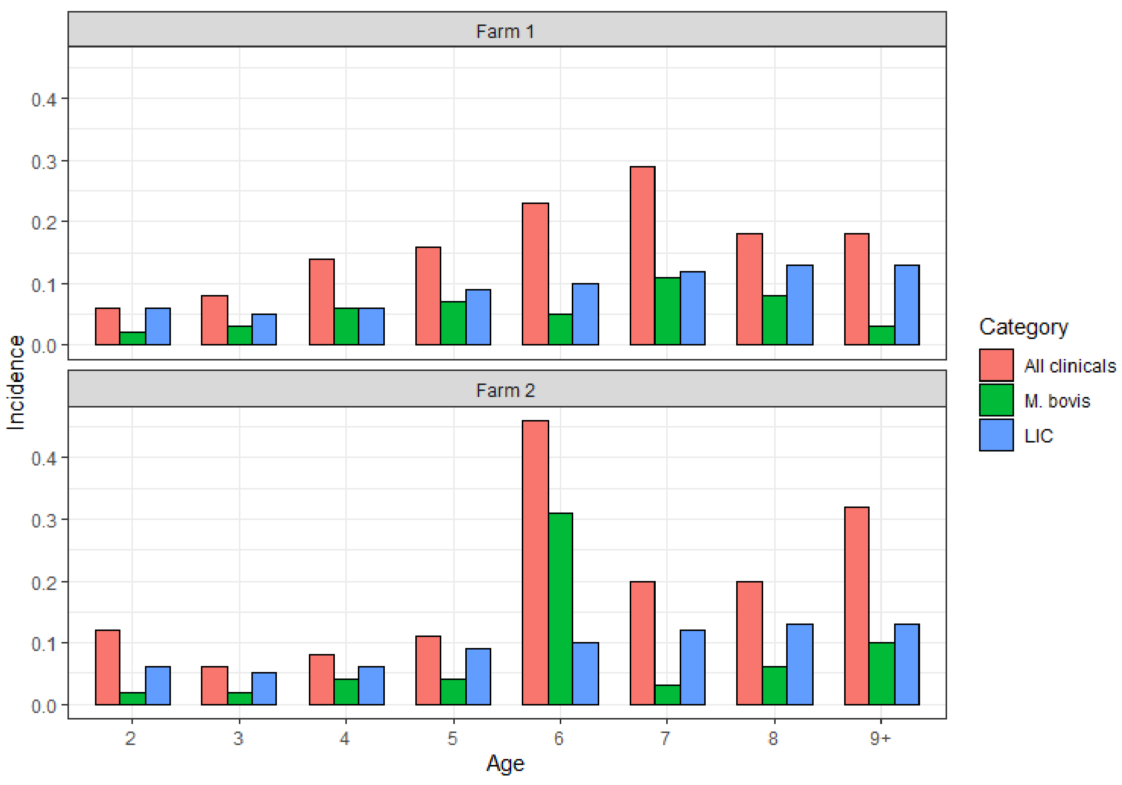


Figure 4-10 Seasonal incidence until depopulation by farm and age group of clinical mastitis (in red), M. bovis clinical mastitis (in green) and national level (in blue)[1]

[1]LIC (2021) data presented by age (in years), for seasonal incidence for 2018 season for CM for herds that recorded CM. Data represents over 196,000 cows with CM data recorded, from < 2.5 million cows in herds that recorded CM. (Livestock Improvement Corporation, 2021).

4.2.6 Subclinical Intramammary Infection (IMI)

As part of the prospective cohort study on Farm 1, 133 CMS were collected on 10.1.2019, from non-clinical, asymptomatic cows in an infected herd. These milk samples were processed by *M. bovis* qPCR at AHL. *Mycoplasma bovis* was detected in one of the 133 samples. The heifer, Tag Id.1068, had received a single course of treatment (ATS) for left hind mastitis during the spring. The course of treatment had started on the 14.8.2018. She had no other animal health record.

From this limited data set, the apparent prevalence of *M. bovis* subclinical IMI in this infected herd on 10.1.2019, was 0.75% (1/133) of cows.

4.2.7 Calf Surveillance

At the first Programme surveillance visit to each farm, and some of the subsequent surveillance visits, up to 40 calves had nasal swabs taken for *M. bovis* qPCR. Different calves were tested at subsequent visits to Farms 3 and 4. Calves were also surveillance tested at slaughter.

Table 4-15 Visit dates for on-farm surveillance calf nasal swabs for M. bovis qPCR, and slaughter surveillance for palatine tonsil swabs qPCR and M. bovis serum ELISA

	On-Farm Surveillance		At Slaughter Surveillance		
		Proportion qPCR Nasal Swab detected (%)		**Proportion qPCR Tonsil Swab detected (%)**	**Proportion Serum ELISA test positive (%)**
Farm 1	18.9.2018	5/39 (13)	23.11.2018 [1]	51/74 (69)	69/74 (93)
Farm 2	18.9.2018	34/34 (100)	30.11.2018/ 3.12.2018 [2]	60/70 (86)	68/70 (97)
Farm 3	19.9.2018	0/40 (0)	26.01.2019	0/100 (0)	0/100 (0)
	15.10.2018	0/40 (0)			
	22.11.2018	0/40 (0)			
Farm 4	19.9.2018	0/40 (0)			
	12.10.2018	0/40 (0)			

[1] Farm 1 Slaughter 23.11.2018 two slaughter events recorded - 30 calves and 44 calves.

[2] Farm 2 Slaughter 30.11.2018 30 calves, and 3.12.2018 40 calves

Over three surveillance testing episodes, no *M. bovis* was detected from nasal swabs from calves on Farms 3 and 4, whereas *M. bovis* was detected in calves from both Farms 1 and 2, which had outbreaks of *M. bovis* CM. Also for 100 Farm 3 calves at slaughter, tonsil qPCR were negative as were serum ELISA.

4.3 Quarter-level Results

4.3.1 Quarter Milk Samples from CM Cows Delivered to the Clinic from Farm 1, 2 and 3

Table 4-16 Proportion of farmer-selected and sampled quarter milk samples (QMS) from cows with clinical mastitis (both apparent treatment failures and apparent treatment success) delivered to the clinic from study Farms 1-3 that had M. bovis detected by qPCR tests

						Proportion quarter qPCR detected (%)[1]	**Nos. of cows sampled**
Farm 1	5.12.2018 0/8	13.12.2018 0/5	18.12.2018 0/8	7.1.2019 0/7	8.1.2019 2/8[2]	2/36 (6%)	32
Farm 2	7.12.2018 0/7	24.12.2018 1/4	7.1.2019 2/5	9.1.2019 5/5	11.1.2019 3/3	11/24 (46%)	22[3]
Farm 3	21.11.2018 0/9	11.12.2018 0/6	13.12.2018 0/10	21.1.2019 1/7		1/32 (3%)	31

[1] Percentage of all QMSs submitted.

[2] Includes two positive *M. bovis* qPCR quarters from same cow that was retested by farmer.

[3] 11 detected quarters from 8 cows

These samples were from later in the outbreak, prior to slaughter. On Farm 1 and 2, the proportion of qPCR positive quarters was small and numerically less than early in the outbreak, when more ATF cows were tested. The proportion of *M. bovis* positive quarters from Farm 2 samples was much greater than those from Farm 1, from which only two quarters (including one previously confirmed *M. bovis* CM cow) tested qPCR positive, with *M. bovis* not detected in the remaining 34 cases of CM.

4.3.2 Other Pathogens Present in Milk with M. bovis

On Farms 1 and 2, QMS were taken from CM cows during diagnostic visits, with some cows having more than one mastitic gland individually sampled. There was a range of times relative to treatment when the samples were taken in cows undergoing treatment, in their milk WHP, or more commonly post treatment. The milk samples from these CM cows, which were sent to Gribbles, Palmerston North, for *M. bovis* qPCR analysis, were also analyzed by a DNA Mastitis Pathogen Multiplex qPCR panel (Qiagen Rotor-Gene Q PCR platform; Qiagen, Hilden, Germany) for *S. aureus*, *S. uberis*, *S. dysgalactiae* and *S. agalactiae*.

Table 4-17 Other pathogens present in QMS M. bovis positive CM on a gland level, for study Farms 1 and 2

	Farm 1 50 quarters (41 cows)	Farm 2 42 quarters (37 cows)
Proportion *M. bovis* only (%)	32/50 (64)	29/42 (69)
Proportion *M. bovis* and *S. uberis* (%)	13/50 (26)	8/42 (19)
Proportion *M. bovis and S. aureus* (%)	3/50 (6)	3/42 (7)
Proportion *M. bovis* and *S. dysgalactiae* (%)	0/50 (0)	0/42 (0)
Proportion *M. bovis* and *S. agalactiae* (%)	0/50 (0)	0/42 (0)
Proportion *M. bovis* and two other pathogens (%) [i]	2/50 (4)	1/42 (2)
Proportion *M. bovis* and three other pathogens (%) [ii]	0/50 (0)	1/42 (2)
Total Proportion *M. bovis* and other pathogens (%)	18/50 (36)	13/42 (31)

[i] *S. aureus* and *S. uberis*
[ii] *S. aureus*, *S. uberis* and *S. dysgalactiae*

Farms 1 and 2 had similar proportions of QMS from CM cows from which only *M. bovis* PCR was detected (64% compared with 69%, respectively). *Streptococcus uberis* was the most common pathogen isolated with *M. bovis*.

4.3.3 Clinical Presentation of M. bovis CM During Outbreak

While a full description of the on-farm management strategies and outcomes of an *M. bovis* CM outbreak on Farms 1 and 2 are outside the objectives of this thesis, a number of findings with respect to CM at a quarter level are reported. These include:

(i) the number of quarters treated at first treatment (4.3.3.1),
(ii) spread to a second quarter (4.3.3.2),
(iii) further spread to three of four quarters (4.3.3.3),
(iv) development of light quarters (4.3.3.4)
(v) the gross examination of infected *M. bovis* milk (4.3.3.5) and.
(vi) antimicrobial therapy (4.3.3.6)

At the clinical farm visits, Table 3-3, where possible, quarters of CM cows were stripped, mastitis treatment records reviewed, and udders palpated.

4.3.3.1 Number of Quarters Treated at First Treatment of M. bovis CM cows

Table 4-18 Number of quarters recorded as treated at first treatment of clinical mastitis for Farms 1 and 2

	No. of cows	**One quarter**	**Two quarters**	**Three quarters**	**Four quarters**	Proportion of one quarter treated (%)
Farm 1	48	42	4	-	2 [1]	42/48 (88)
Farm 2	42	38	4[2]	-	-	38/42 (90)

[1] Farm 1 treated six cows with parental Tylan 200 injection for multiple quarter mastitis. Four cows were recorded as two quarter mastitis and two cows were recorded as four quarter mastitis.
[2] Similarly, Farm 2 treated two cows with parental Tyan 200 injection and were recorded as two quarter mastitis.

4.3.3.2 *Spread or Movement to a Second Quarter in Udder*

Later in the outbreak some CM cows only received one course of mastitis treatment, and if there was not a clinical cure as determined by the farmer, these were deemed ATF and received no further treatment. Consequently, the spread of mastitis was not recorded in some cows. Table 4-19 reports data that was recorded.

Table 4-19 Spread to a second quarter in M. bovis CM diagnosed cows

			For second quarter treated			
	Start as single quarter	Proportion with treatment of a second quarter (%)	Same side	Diagonal	Across udder	Proportion with spread to second quarter on same side (%)
Farm 1	42	28/42 (67)	23	3	2	23/28 (82)
Farm 2	38	27/38 (71)	20	2	4	20/27 (74)

On Farm 1, 82% of the recorded *M. bovis* CM cows which had second quarter treatment, was on the same side of the udder as the first case. On Farm 2, this was 74%.

4.3.3.3 *Spread to 3 or 4 Quarters*

As in 4.3.3.2, the spread to 3 or 4 quarters maybe under-reported as treatment ceased on some cows, and some CM cows were slaughtered early in the outbreak (once outside meat WHP).

Table 4-20 Spread of clinical mastitis in M. bovis diagnosed cows to 3 or 4 quarters

	No. of cows starting with 1 or 2 quarter mastitis	**Developed 3 or 4 quarter mastitis**	**Proportion with 3 or 4 quarter mastitis (%)**
Farm 1[1]	46	7	7/46 (15)
Farm 2	42	12	12/42 (29)

[1] Data on Farm 1 incomplete. Of the 36 cows examined at Visit 2 on 24.10.2018 (Table 3-3), only 18 ATF cows were physically examined at Visit 3 on 22.11.2018. Mastitic cows were dried off earlier on Farm 1 hence treatment and record keeping ceased.

4.3.3.4 Development of Light Quarters in M. bovis CM cows

Table 4-21 Light (agalactic) quarters and gross udder findings in M. bovis CM cows

	No of cows' udders palpated (at least once)	Number of agalactic quarters				Proportion of cows with one agalactic quarter (%)	Other findings
		One quarter	Two quarters	Three quarters	Four quarters		
Farm 1	40	15	5			15/40 (38)	3 ruptured udder abscesses through the skin 1 hard quarter
Farm 2 [1]	34	14	3	2		14/34 (41)	2 ruptured udder abscesses through the skin 2 hard quarters

[1] On Farm 2, 27 CM cows were palpated on 17.10.2018 at Visit 1, and then 30 mastitis cows were presented at Visit 2 on 13.11.2018, 20 of these had been presented previously. More in milk cows on Farm 2, than Farm 1, were able to be followed, closer to the 'peak' of the *M. bovis* CM outbreak.
On Farm 2, none of the eight mastistic cows diagnosed as qPCR milk positive, from milk that were delivered into the clinic by the farmer later in the outbreak, were palpated.

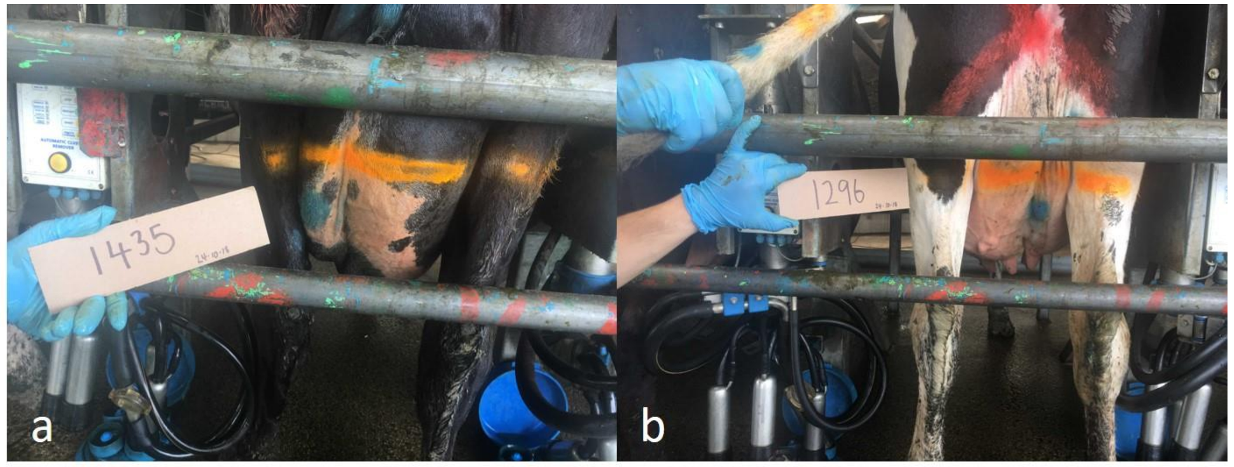


Figure 4-11 Farm 1. Two cows presented with light quarters.
(a) Tag Id.1435 left hind and (b)Tag Id.1296 right hind

4.3.3.5 Gross Examination of QMS from M. bovis CM Cows

The appearance of infected *M. bovis* milk varied in colour, consistency and overall gross appearance of the exudate present.

Figure 4-12 Mycoplasma bovis was detected in all samples. (a), (b) and (e) Farm 1. (c) and (d) Farm 2

4.3.3.6 *Antimicrobial Therapy for M. bovis CM Cows*

Farms 1, 2 and 3 milked their antibiotic-treated cows twice a day (TAD), with Farm 1 and 2 treating cows TAD or once a day (OAD) as per label instructions, while Farm 3 only treated OAD (in the morning).

Table 4-22 The number of mastitis treatments for M. bovis CM cows on Farms 1 and 2

	Average no. Intramammary syringes/quarter	**Average no. Intramammary syringes/cow**	**No. of cows Intramuscular treatments concurrently (Tylan/Mamyzin)**
Farm 1	7	8.5	16 (10/6)
Farm 2	13	17.5	4

An *M. bovis* CM quarter on Farm 2 received nearly double the number of intramammary syringes/infected quarter (13 syringes), compared to Farm 1 (7 syringes). There was a high use of antibiotics on *M. bovis* CM quarters.

Part 2: Agreement Study

4.4 Agreement Study Between Serum ELISA SP% and Tonsil Swab in Both Clinical and Non-clinical Surveillance Slaughter Cows

On Farm 1 and Farm 2, 39 and 26 *M. bovis* CM cows, respectively, which were sampled on-farm and had both serum ELISA and palatine tonsil qPCR swab results, were tracked at slaughter and sampled once again to determine both serum ELISA and palatine tonsil swab results.

From surveillance slaughter testing, for Farm 1 82 cows had serum ELISA and palatine tonsil qPCR swab results. For Farm 2, the three surveillance slaughter dates over four days, provided 226 cows with paired data. As previously noted, the prospective cohort study data (3.11/6.1.4) from Farm 1, with 15 days between on-farm serum ELISA (10.1.2019) and tonsil qPCR swabbing at slaughter (25.1.2019) has not been included in this Agreement study. Of note though, 40/100 cows from the study were tonsil qPCR positive. There were operational difficulties at Farm 3 slaughter surveillance, so this data has not been included either.

4.4.1 Two by Two Tables for Analysis of Palatine Tonsil Swabs for M. bovis qPCR and M. bovis Serum ELISA

4.4.1.1 Mycoplasma bovis CM cows sampled on-farm and at slaughter for Farm 1 and 2

(1) Farm 1 (39 Cows)

a. On farm 23.11.18

	Tonsil Det (%)	Tonsil ND (%)	Total (%)
SP +ve	32/39 (82)	5/39 (13)	37/39 (95)
SP -ve	1/39 (3)	1/39 (3)	2/39 (5)
	33/39 (85)	6/39 (15)	39/39 (100)

b. At slaughter 4.12.18 (25 cows)/17.12.18 (14 cows)

	Tonsil Det (%)	Tonsil ND (%)	Total (%)
SP +ve	25/39 (64)	10/39 (26)	35/39 (90)
SP -ve	3/39 (8)	1/39 (3)	4/39 (10)
	28/39 (72)	11/39 (28)	39/39 (100)

(2) Farm 2 (26 cows)

a. On farm 28.11.18

	Tonsil Det (%)	Tonsil ND (%)	Total (%)
SP +ve	24/26 (92)	0/26 (0)	24/26 (92)
SP -ve	2/26 (8)	0/26 (0)	2/26 (8)
	26/26 (100)	0/26 (0)	26/26 (100)

b. At slaughter 18.12.18 (25 cows)/7.1.19 (1 cow)

	Tonsil Det (%)	Tonsil ND (%)	Total (%)
SP +ve	19/26 (73)	6/26 (23)	25/26 (96)
SP -ve	0/26 (0)	1/26 (4)	1/26 (4)
	19/26 (73)	7/26 (27)	26/26 (100)

4.4.1.2 Farm 1 and Farm 2 Mycoplasma bovis CM Cows Combined Data

a. On farm

	Tonsil Det (%)	Tonsil ND (%)	Total (%)
SP +ve	56/65 (86)	5/65 (8)	61/65 (94)
SP -ve	3/65 (5)	1/65 (2)	4/65 (6)
	59/65 (91)	6/65 (9)	65/65 (100)

b. At slaughter

	Tonsil Det (%)	Tonsil ND (%)	Total (%)
SP +ve	44/65 (68)	16/65 (25)	60/65 (92)
SP -ve	3/65 (5)	2/65 (3)	5/65 (8)
	47/65 (72)	18/65 (28)	65/65 (100)

4.4.1.3 Surveillance of Non-clinical Cows at Slaughter for Farm 1 and Farm 2

Farm 1 29.1.2019 - 82 cows

	Tonsil Det (%)	**Tonsil ND (%)**	**Total (%)**
SP +ve	36/82 (44)	19/82 (23)	55/82 (67)
SP -ve	3/82 (4)	24/82 (29)	27/82 (33)
	39/82 (48)	43/82 (52)	82/82 (100)

Farm 2 7.1.2019 – 11.1.2019 - 226 cows

	Tonsil Det (%)	**Tonsil ND (%)**	**Total (%)**
SP +ve	104/226 (46)	58/226 (26)	162/226 (72)
SP -ve	9/226 (4)	55/226 (24)	64/226 (28)
	113/226 (50)	113/226 (50)	226/226 (100)

4.4.2 Agreement Between Serum ELISA and Palatine Tonsil qPCR on Farms 1 and 2

The observed agreement, Gwet AC1 values and judgement are presented in Table 4-23.

Table 4-23 Observed Agreement and Gwet AC1 Value for Clinicals and Surveillance Slaughter Cows

		Observed Agreement	**Gwet AC1**	**Gwet AC1 Confidence Intervals (C.I.)**	**Judgement (agreement)**
***M. bovis* CM cows**	Farm 1 (on farm)	0.85	0.81	0.65 - 0.98	Near perfect
	Farm 1 (slaughter)	0.67	0.52	0.23 - 0.80	Moderate
	Farm 2 (on farm)	0.92	0.92	0.79 – 1.00	Near perfect
	Farm 2 (slaughter)	0.77	0.69	0.41 - 0.97	Substantial
***M. bovis* CM cows combined**	Farm 1 & 2 on farm	0.88	0.86	0.75 – 0.96	Near perfect
	Farm 1 & 2 at slaughter	0.70	0.59	0.39 – 0.79	Moderate
Surveillance of cows at slaughter	Farm 1	0.73	0.48	0.28 - 0.67	Moderate
	Farm 2	0.70	0.43	0.31 - 0.56	Moderate

Descriptive Statistics for Agreement study

For *M. bovis* CM cows the Observed Agreement was higher on-farm for Farm 1 and Farm 2 than at slaughter with on-farm being 0.85 and 0.92 and at slaughter 0.67 and 0.77, respectively. The Gwet AC1 were also higher on-farm being 0.81 and 0.92 on farm and then 0.52 and 0.69 at slaughter. This difference was also noted when the farms were combined, with on-farm being 0.86 and slaughter 0.59.

At surveillance slaughter, the Observed Agreement for the two groups considered were similar. The Gwet AC1 value at 0.48 (Farm 1) and 0.43 (Farm 2) showed moderate agreement.

Part 3: Pathological Findings

4.5 Morphological Patterns of Mammary Gland Lesions in Dairy Cows with *M. bovis* Clinical Mastitis

4.5.1 Gross Pathology

The udders of 14 known *M. bovis* CM cows were grossly examined at slaughter by the author (KPK). This represented 55 quarters, as one cow (Tag Id.1435; E533-18) had the left front quarter trimmed off prior to examination. Overall, there was marked variation in the severity of the gross lesions present, the estimated percentage of mammary parenchyma affected, the type of lesions present, and the volume and consistency of exudate present (Fig. 4-13). Lesions were noted in multiple quarters in 13 of the 14 cows, with three quarters affected in three cows and four quarters affected in seven cows.

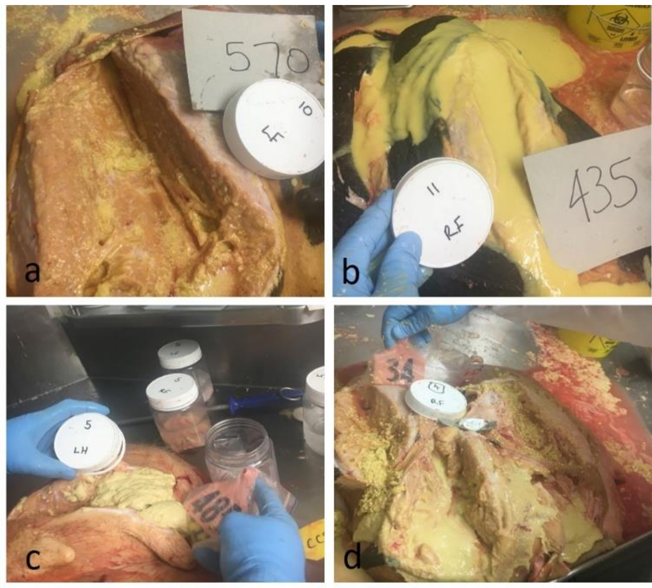


Figure 4-13 Mammary glands of cows affected with M. bovis clinical mastitis - exudates.
The amount and appearance of the exudate present on the mammary parenchyma varied from flocculent yellow-tinged (a), to abundant, thick yellow-tinged exudate (b and c). In some glands, the appearance of the exudate varied in each affected quarter (d).

Gross lesions in the mammary parenchyma (fresh specimens) were characterised by disruption of the parenchyma by large, cavitated areas of abscessation, multifocal to coalescing areas of tissue fibrosis (Fig. 4-14), caseous necrosis (Fig. 4-15), and cystic dilation of the mammary parenchyma (Fig. 4-16). As previously noted, the severity of these lesions varied amongst affected quarters, with some quarters having less than 10% of the quarter affected and some up to 80% of the quarter affected. Due to the difficulties associated with tissue collection, a quantitative analysis was not performed on fresh tissues.

Gross examination of fixed mammary gland tissue was conducted to determine the presence/absence of the most common gross lesions noted during inspection at slaughter. The three most common morphological changes present in these specimens were like those described for fresh specimens and included parenchymal fibrosis (51/52) (Fig. 4-14), multifocal to coalescing areas of caseous necrosis (46/52) (Fig. 4-15), and cystic dilation of the mammary parenchyma (46/52) (Fig. 4-16). A summary of these results is presented in Table 4-24.

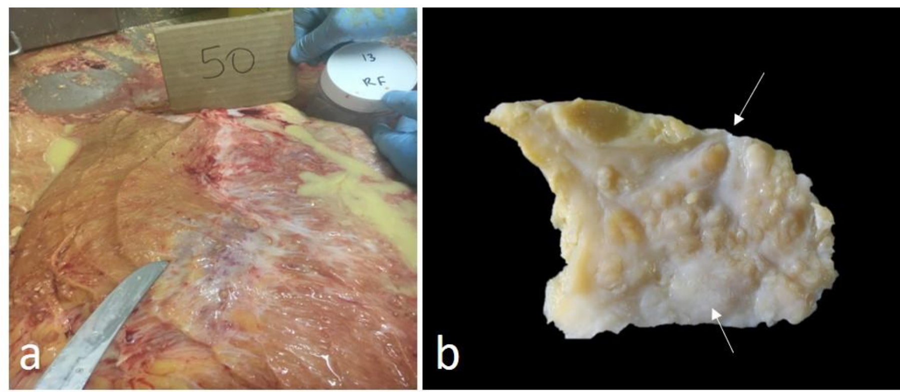


Figure 4-14 Mammary glands of cows affected with M. bovis clinical mastitis – tissue fibrosis
(a) Loss of glandular tissue and replacement by multifocal to coalescing areas of tissue fibrosis (fresh specimen) (Tag Id.50; E541-18), (b) The mammary parenchyma is obliterated by fibrous tissue (arrows) (fixed specimen) (Tag Id.557; E536-18).

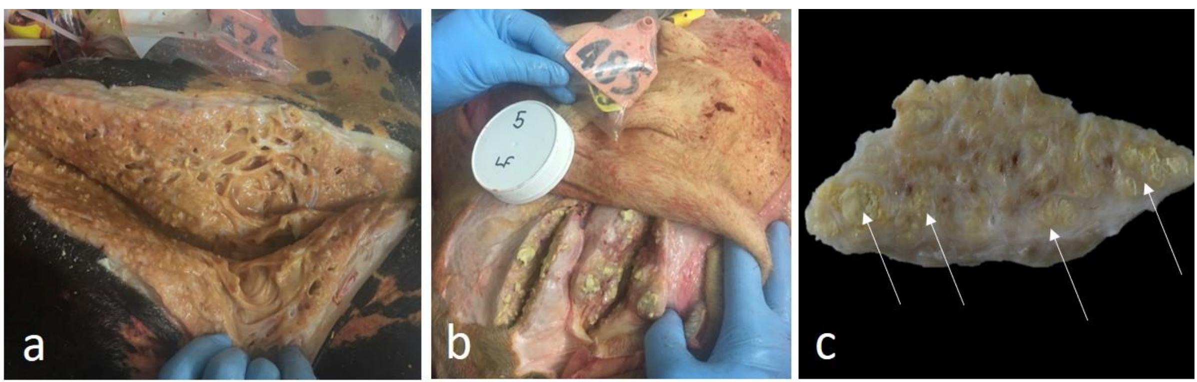


Figure 4-15 Mammary glands of cows affected with M. bovis clinical mastitis – caseous necrosis
(a and b) Multifocal to coalescing areas of caseous necrosis (fresh specimens) (Tag Id.426; E534-18 and Tag Id 485; E532-18 respectively), (c) Areas of caseous necrosis (arrows) (fixed specimen) (Tag Id. 485; E532-18).

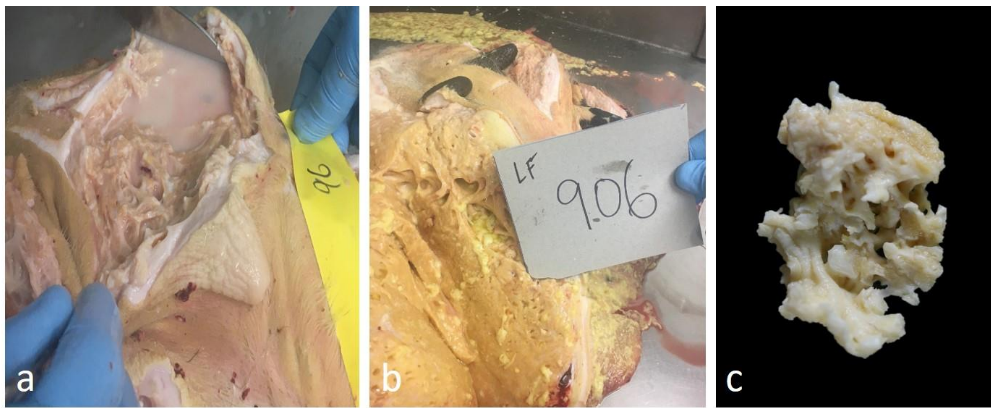


Figure 4-16 Mammary glands of cows affected with M. bovis clinical mastitis – cystic dilatation
(a and b) Multifocal to coalescing areas of cystic dilation (fresh specimens) (Tag Id.1296; E530-18 and Tag Id 906; E528-18 respectively), (c) Marked cystic dilation of the mammary parenchyma (fixed specimen) (Tag Id. 1296; E530-18).

4.5.2 Histopathology

Histologically, inflammatory lesions were present in 92.7% of sections examined (51/55). The predominant inflammatory patterns were granulomatous (18/55), suppurative (15/55), pyogranulomatous (8/55), lymphoplasmacytic (7/55), mixed (2/55), and lymphocytic (1/55). No inflammation was present in four sections. Other common lesions included the presence of fibrosis (50/55), ductal dilation (45/55), and caseous necrosis (23/55) (Fig. 4-17).

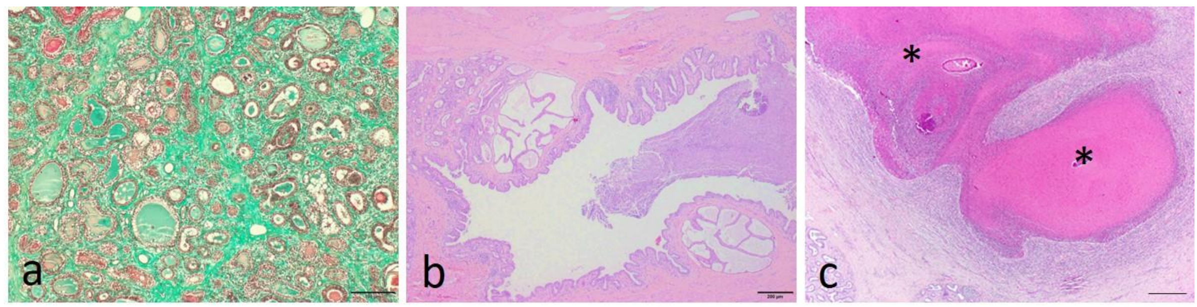


Figure 4-17 Mammary glands of cows affected with M. bovis clinical mastitis – histopathology (a) Interstitial fibrosis with glandular atrophy (Tag Id.485; E532-18) (Masson's Trichrome stain 20X). (b) Ductal dilation (Tag Id.594; E537-18) (H&E 4X) (d) Multifocal to coalescing areas of caseous necrosis (Tag Id.485; E532-18) (H&E 4X).

Gram stains were done on 41 sections displaying granulomatous, pyogranulomatous or suppurative inflammatory lesions. Two sections from the same cow (Tag Id.655; E542-18) had Gram-positive cocci within the necrotic material. Ziehl-Neelsen stain was done on 23 sections with evidence of granulomatous inflammation; all sections were negative for acid-fast positive organisms.

4.5.3 Immunohistochemistry

Of the 55 mammary gland samples submitted for IHC, 55% (30/55) were immunoreactive for *M. bovis* antigen and 4% (2/55) yielded a "suspicious" result. Of the positive samples, 87% (26/30) were classified as granulomatous or pyogranulomatous on histology. The remaining immunoreactive samples (13% or 4/30) were classified as a suppurative mastitis. All 30 mammary gland samples identified as positive consistently exhibited strong immunolabeling for *M. bovis* antigen within foci of caseous necrosis (Fig. 4-18).

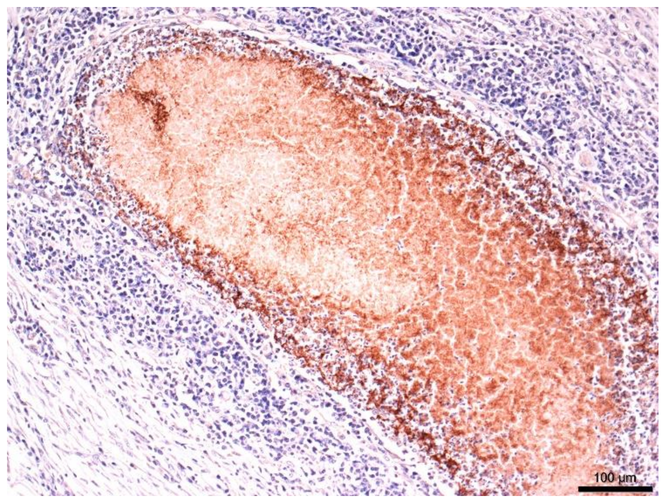


Figure 4-18 Immunohistochemistry for M. bovis antigen
Mammary gland of cow affected with M. bovis clinical mastitis (Tag Id.426; E534-18) with strong M. bovis immunolabeling (red-brown staining) in foci of caseous necrosis, surrounded by large numbers of inflammatory cells (H&E 10X with DAB counterstain).

A summary of the histological lesion characterisation and immunohistochemistry results are presented in Table 4-24. Full data is presented in Appendix Document 6 Chapter 4, Table 6-6.

Table 4-24 Description of gross lesions, Histological lesion characterization, Special stains and Immunohistochemistry from 55 M. bovis clinical mastitis quarters at slaughter.

Gross Lesions[1]		Histological Lesion Characterisation[2]		Special Stains Gram Stain	Immumohistochemistry
	Proportion Positive Samples (%)	Predominant morphologic pattern	Proportion Positive Samples (%)	Proportion Positive Samples (%)	Proportion Positive Samples (%)
		Inflammation			
Fibrosis	51/52 (98)	Granulomatous	18/55 (33)	2/41 (5)	30/55 (55)
Cystic Dilation	46/52 (89)	Suppurative	15/55 (27)		
Caseous Necrosis	41/52 (79)	Pyogranulomatous	8/55 (15)		
		Lymphoplasmacytic	7/55 (13)		
		No inflammation	4/55 (7)		
		Mixed	2/55 (4)		
		Lymphocytic	1/55 (2)		
		Other lesions			
		Fibrosis	50/55 (91)		
		Ductal dilation	45/55 (82)		
		Caseous necrosis	23/55 (42)		

[1] From 14 cows (56 quarters) 1 quarter was trimmed off on slaughter board [cow #6 (Tag Id.1435) LF]. There was no fixed specimen photo for 3 quarters [cow #2 (Tag Id.329) RH]; [cow #9 (Tag Id.594) RH]; [cow #10 (Tag Id.570) RF].

[2] From Leah Cristina Gans, Masters in Veterinary Studies 2020 “Morphologic and immunohistochemical characterisation of *Mycoplasma bovis* mastitis in dairy cows in New Zealand” (Gans, 2020)

Chapter 5 Discussion

5.1 Introductory Comments and Key Results

This case study gave a unique opportunity to describe an outbreak of *M. bovis* CM across a large-scale, pasture-based, seasonal-calving, North Otago (NO) dairy farming enterprise, for which analysis of Programme data had identified a hypothesized infection source and date to the dairy farms. With the NZ government decision in May 2018 to eradicate *M. bovis* from NZ (New Zealand Government, 2018), the three infected dairy farms in this operation were depopulated within five months of the first diagnosis of *M. bovis,* by BTM qPCR testing on 30.8.2018. Consequently, the outbreak could only be followed for this period and impending depopulation overshadowed some on-farm management decisions.

Several of the key epidemiological, clinical and pathological findings from this outbreak have not been previously published. These findings include: herd-level BTM PCR test results, with over 50 for each of Farms 1-4, and BTM ELISA test results, with over 30 tests for each of Farms 1-3, from spring (late August 2018) to depopulation in summer (January/Feb 2019); the estimation of true infection prevalence from serum ELISA test results across the four study farms; and the reporting of three diagnostic tests for individual *M. bovis* CM cases, namely QMS qPCR, *M. bovis* serum ELISA, and palatine tonsil qPCR. A model of the mean serum ELISA SP% for *M. bovis* CM cases from two infected farms, from the time of milk qPCR diagnosis, was also proposed.

Other novel aspects of this thesis are the description of the clinical presentation at a quarter level of CM cases including an evaluation of other pathogens detected in QMS samples; a description of the agreement between two diagnostic tests, namely serum ELISA and qPCR palatine tonsil, in both *M. bovis* CM cows on-farm and at slaughter, and non-clinical slaughter surveillance cows; and the morphological patterns of mammary lesions of *M. bo*vis CM cases, together with histopathological lesion characterization and immunoreactivity for *M. bovis* antigen, to support the view that *M. bovis* was the causative agent for this CM outbreak.

The Farming Operation, Associated Risks and Subsequent Outbreak of M. bovis CM

Many of the KPIs and management practices on this high-input, high-producing dairy operation (Chapter 3) are similar to other large dairy herds in NO. However, a number of farming practices warrant noting with respect to potential risk of disease and specifically the risk of an *M. bovis* outbreak on Farms 1 and 2 in this operation. It is hypothesised that *M. bovis* is an opportunistic pathogen that needs certain circumstances to induce clinical disease (Jasper, 1981b).

These risks include herd size, with herd size >500 being associated with increased prevalence of mycoplasmas in BTM samples (McCluskey et al., 2003). They also include stock trading, stock movements and grazed out stock (Aebi et al., 2015; Burnens et al., 1999; Punyapornwithaya et al., 2010). The high number of Participant Codes in these four herds (118, 73, 77 and 84) reflected a significant historic trading history. This highly integrated farming operation had a high number of reported stock movements and grazed out stock.

This outbreak occurred during the spring calving season, where calving is a common stress factor (Bushnell, 1984) and cause of host immunosuppression (Bayoumi et al., 1988; Boothby et al., 1986) that may predispose clinical *Mycoplasma* spp. outbreaks. The policy of batch drying off cows in May-July, with the last group of late calving cows being dried off coinciding with the early calving period of the following season, means that if infected cows were grazed or milked with uninfected, freshly calved cows, this co-mingling would increase the risk of both direct and indirect transmission of *M. bovis* (Calcutt et al., 2018; Punyapornwithaya et al., 2011). Additionally, farm history of high numbers of treatments for CM and chronic *S. aureus* mastitis in these herds, may have been risk factors, as other concomitant diseases e.g. metritis and clinical ketosis have been deemed risk factors for herd-level presence of *M. bovis* (Aebi et al., 2015).

5.2 Herd-level Investigations

5.2.1 Bulk Tank Milk qPCR and Bulk Tank Milk ELISA

Bulk Tank Milk qPCR and BTM ELISA tests were carried out on all four study farms, during this outbreak and offer a comprehensive data set of multiple testing not previously reported in the literature. While both BTM tests are validated tests, they are not gold standards tests (Nielsen et al., 2015), but their values can be used to identify *M. bovis* infection at a herd-level and not just *M. bovis* infection at a cow-level (Cai et al., 2005; Nielsen et al., 2015; Parker, House, Hazelton, Bosward, Morton, et al., 2017).

On Farm 1, 43/52 (83%) of the BTM samples tested *M. bovis* PCR positive from 52 sample days. On Farm 2, 20/50 (40%) of samples from 50 sample days were positive. In contrast, neither Farm 3 nor Farm 4 returned a BTM PCR positive result over 54 and 85 sample dates, respectively. Hazelton et al. (2020) note that a single cow infected with *M. bovis* (low to moderate PCR CT value), would likely turn the average Australian herd of 270 cows, BTM qPCR positive. A Finnish study (Vähänikkilä et al., 2019) sampled 19 recently-infected farms over a two-year period, and found only 7/263 (3%) BTM qPCR positive samples. Six of these seven samples, collected from five farms (26% of the herds) were collected within four weeks of the herd index mastitis case. The high number of BTM samples that were qPCR positive on Farm 1 and 2 suggest there was continual shedding of *M. bovis* in milk from non-detected clinical or subclinically infected cows, even after cows diagnosed with *M. bovis* CM cows were dried off and removed from both the milking herd and red herd.

For the BTM ELISA, Farm 1 had 29/32 (94%) positive tests, and Farm 2, 27/30 (97%) positive tests. After three negative BTM ELISA tests on Farm 3, the first positive BTM ELISA on 24.10.2018, occurred on the same day that the herd was deemed herd-level serology positive by the Programme. After this first positive BTM ELISA on Farm 3, 21/27 (78%) of the tests were positive. Farm 4 remained BTM ELISA negative, though only four BTM ELISA tests were reported. Australian work showed their BTM ELISA ODC % was highest in the first eight months following the initial outbreak of *M. bovis* (Parker, House, Hazelton, Bosward, Morton, et al., 2017) and significantly higher 5-8 weeks after the start of a seasonal calving period (Parker et al., 2018). While the NO outbreak occurred during the spring calving period, the herds could not be followed over a similar extended period due to depopulation and only two BTM ELISA samples were taken in the calving period, the highest BTM ELISA SP% value was reported during this period (Farm 1 on 25.9.18 and Farm 2 on 26.9.18).

The number of days when both BTM PCR and BTM ELISA were simultaneously positive in this outbreak was high, with 23/31 (74%) days on Farm 1 and 13/29 (45%) on Farm 2. In contrast, a 100-farm

prevalence study in Belgium, which identified seven farms as PCR positive and 17 farms as antibody ELISA positive (Gille et al., 2018) found no overlap between PCR- and ELISA-positive farms. Australian work (Parker, House, Hazelton, Bosward, Morton, et al., 2017) from 192 samples identified 74 BTM ELISA postives and seven qPCR positives. Only three of these seven positive qPCR samples were ELISA positive. They concluded that previous exposure to *M. bovis* was much more common than shedding of the bacterium in milk. If this is so, the presence of both bacterial DNA and antibodies in the BTM sample at the same time is unlikely. A large Danish study also found very low agreement (Nielsen et al., 2015) where using the manufacturers recommendation for BTM ELISA cut-off value, only 4/490 (0.8%) antibody-positive northern herds, and 21/2871 (0.73%) antibody-positive southern herds, were also PCR positive. However, in the NO outbreak, there was a high proportion of simultaneously positive days on Farms 1 and 2. These results represent samples being taken through the peak of a known *M. bovis* CM outbreak which occured during a spring calving season, where new CM cases were being detected regularly over several weeks. This good herd-level agreement of BTM tests reflects spread of *M. bovis* through the herd (BTM ELISA) with ongoing new CM cases (BTM qPCR). Complementary diagnostic data on herd infection status is provided by paired BTM ELISA and BTM PCR, from different biological standpoints (Parker et al., 2018). The BTM ELISA test appeared more sensitive than the BTM PCR test.

5.2.2 Individual Cow Serum ELISA Testing (aggregated from individual up to herd-level, by farm sampling date)

This study gave a unique opportunity to consider seroconversion *(change in test result for an individual from negative (below cut-off) to positive (above cut-off)),* and seroprevalence (*proportion of population which is seropositive*) in infected dairy herds within the same farming operation. The on-farm and slaughter surveillance blood testing data allowed cows with both known clinical disease and no clinical disease in infected herds to be followed. Herd-level sensitivity (SeH) and herd-level specificity (SpH) are determined by animal-level Se and Sp, number of animals tested, disease prevalence in the herd and the number of individual animals positive (Sergeant & Perkins, 2015a). In this study BTM ELISA was used as a screening test and cow-level serum ELISA interpreted at the herd-level as a confirmatory test, where two rounds of positive herd-level serology, interpreted in series, was the herd case definition. Testing in series increases specificity and decreases sensitivity. The high Se and Sp test performance estimates, of the ID.Vet ELISA, make it a very useful animal-level and herd-level diagnostic test, and mean that a positive PCR result was not required to identify an infected farm.

The distribution of individual cow *M. bovis* serum ELISA SP% values in the four farms (Fig. 4.3), Farms 1-4 over time, indicate rising antibody titres and seroconversion on Farms 1, 2 and 3. This pattern is indicative of recent spread of *M. bovis* in these herds, either by direct transmission or transmission via nose-to-nose contact, or indirect transmission via milking equipment and other fomites (Calcutt et al., 2018; Fox, 2012; Maunsell et al., 2011). The literature offers limited information about individual animal *M. bovis* antibody response to natural infections (Hazelton et al., 2018), in particular there are no data on the antibody response of 'non-clinical' cows in an infected herd (Petersen et al., 2016). The rising antibody titres and seroconversion on these farms highlights active infection in these herds. Individual cow serum ELISA aggregated up to herd-level, by farm sampling date, across one farming enterprise, where two farms had an outbreak of *M. bovis* CM, has not been presented before.

5.2.3 Estimation of True Prevalence

There was a significant trend of increasing estimated true prevalence for Farm 1, 2 and 3 over time, with no evidence of a trend for Farm 4. Prior to herd slaughter, from the data available, Farms 1, 2 and 3 appeared to have similar estimated true prevalence i.e. Farm 1 0.74 (95% CI 0.61 – 0.85), Farm 2 0.86 (95% CI 0.75 – 0.95) and Farm 3 0.76 (95% CI 0.63 – 0.87). The seroprevalence of *M. bovis* in a herd which is experiencing an outbreak of *M. bovis* CM will be affected by numerous factors including firstly, the rate of *M. bovis* transmission between cows, secondly the proportion of cows with IMI due to *M. bovis* and thirdly the diagnostic sensitivity of the method used to detect clinical cases in the herd (Hazelton et al., 2020). Transmission rates are documented, or modelled, for some infectious diseases of cattle e.g. salmonella (Lanzas et al., 2008; Nielsen et al., 2007; Xiao et al., 2005) but there is no published data on transmission rates for herds experiencing *M. bovis* CM outbreaks.

The estimated true infection prevalence on the study farms needs to be considered with the animal movements in the enterprise and animal-level sampling as described in Fig. 3-2. At the first surveillance serum ELISA test, the APs for study Farms 1-4 were 0.33, 0.16, 0.03 and 0.00 respectively. While movement dates and management dates were similar, except for mixing at grazing on Farm 7 (3.5.1) it could be proposed fewer infected heifers returned to Farm 3 platform, from Farm 6 for transition management, compared to Farms 1 and 2. Farm 4 heifers had been grazed on Farm 5, and had no contact with the other heifer lines.

There are limited data points for which true prevalence can be estimated, and on Farm 1 and 2, we only have data at first surveillance on 18.9.18 and then January 2019. These two farms experienced *M. bovis* CM outbreaks, with the prospective cohort study of non-clinical cows on Farm 1 on 10.1.19 having an AP of 0.65 (86/133). Transmission of *M. bovis* on these farms would be expected to be

extensive. For Programme surveillance purposes, some of the cows for Farm 3 on-farm serum ELISA testing, especially at farm surveillance Visit 3, where there was an AP of 0.13 (13/100), were not randomly selected. Some previously positive cows were purposively resampled with the herd seroprevalence proportion biased upwards. Slaughter surveillance on Farm 3, on 1.2.19 faced operational difficulties, where the AP was 0.69 (53/77).

From the author's reading, this is possibly the first reported estimate of true prevalence from seroprevalence data for one farming enterprise with *M. bovis* CM with a single hypothesized infection source and date.

5.2.4 Association Between BTM ELISA and Estimated True Infection Prevalence

For Farm 1 and 2 BTM ELISA decreased from early-mid December, while estimated true prevalence, estimated from individual cow serum ELISA, increased. In contrast, on Farm 3 both BTM ELISA and true prevalence increased with time. The ELISA test used in this outbreak measured IgG, which is produced in the blood and found in lower concentrations in the milk (Parker et al., 2018). Milk produced IgA and IgM were not measured in this study. These differences between Farm 1 and 2 compared with Farm 3 may be explained in part by contrasting farm management practices at the time of the outbreak, including withholding or culling of CM cows with ATF. During the outbreak, confirmed *M. bovis* CM cases on Farm 1 and 2 were steadily removed from the herd and dried off, and slaughtered (with slaughter dates Farm 1 29.10.2018, 4.12.2018, and 17.12.2018 and Farm 2 18.12.2018). These CM cows, with high SP% values were not milked into the vat. It could also be hypothesized, that early in the outbreak, on Farms 1 and 2, there was infection in fewer animals but they had higher individual SP% values and as the outbreak progressed with spread through the herd, individual SP% values tended to drop but a higher proportion of cows seroconverted. The consequence of this shift would be BTM ELISA would decrease and estimated true prevalence would increase.

5.2.5 Bulk Milk Somatic Cell Count

With the confirmed diagnosis of *M. bovis* on Farm 1 and Farm 2, late spring herd testing was cancelled, resulting in no ISCC, or individual production data, for these farms through the outbreak. Published literature has presented differing views on the value of ISCC in *M. bovis* mastitis (Biddle et al., 2003; Ghadersohi et al., 1999; Hazelton et al., 2020). Farm 3 herd testing was subsequently cancelled as well after the first test. The BMSCC graphs (Appendix Document 1 Chapter 4, Fig. 6-1) suggest there is

an issue with mastitis on farm due to unusually increased counts, but further investigation of the factors causing this mastitis needs knowledge of on-farm practices. The BMSCC for Farms 1 and 2, appeared more under control during the spring 2018 outbreak of *M. bovis* of 2018, compared to the previous season. However, numerous farm-level mastitis control measures had been implemented during the autumn/winter of 2018. These included a high culling rate of chronic mastitis cases (the author assisted with cull lists), the wide use of long-acting dry cow therapy (DCT) and the use of teat sealants in heifers for the first time. The outbreak of *M. bovis* CM did not appear to have an impact on BMSCC, however, as cows with *M. bovis* CM were diagnosed, or were deemed ATF by the farmer, they were removed from the milking herd and would not have contributed to the BMSCC post removal.

Other considerations-BVDV

From BTM monitoring for BVDV through LIC (Table 3-1) and blood testing of individual *M. bovis* CM cows (Table 4-8) for BVDV Ag ELISA, there was no evidence of circulating BVDV on Farms 1 and 2 throughout the outbreak of *M. bovis* CM, though the herds had been exposed at some stage. The synergism between BVDV and *M. bovis* pneumonia and arthritis which was proposed by Canadian researchers (Shahriari et al, 2002), has not been widely explored in *M. bovis* CM outbreaks.

5.3 Animal-level Investigations

5.3.1 Mycoplasma bovis CM Cases and SP% Values

On Farms 1 and 2, farmer-selected cases of CM, predominantly ATF cases, during this outbreak were presented to the veterinarian (KPK) for sampling. These CM cases were tested by QMS *M. bovis* qPCR, individually blood tested for *M. bovis* serum ELISA (except for Farm 1 visit on 18.9.18) and palatine tonsils swabbed for *M. bovis* qPCR test (see Agreement study). Serum ELISA SP% for 44 *M. bovis* CM cows on Farm 1 and 39 cows on Farm 2 that could be traced through the outbreak, were individually plotted over time, and offered a unique data set for analysis.

The analysis of serum ELISA SP% values in individual CM cows, for this study, may aid the clinical veterinarian in the diagnosis of *M. bovis* CM. The serum ELISA results and plots of individual CM cows' SP% (Fig. 4-6) show for Farm 1 8/44 cows (18%) and similarly for Farm 2 7/39 cows (18%) were less than SP% = 60, at the time of diagnosis, but rose rapidly after that. The smoothed function for the combined model (4.2.3.3), indicates that on average cows with *M. bovis* CM will be serum ELISA test

positive (SP% ≥ 60) at a mean of 5 days after the initial clinical diagnosis, but could have been as few as -2 and as many as 7 days following initial diagnosis. The reported IP (period of time from infection to the onset of clinical signs) for *M. bovis* CM is dependent on a number of factors (Calcutt et al., 2018) but range from 2-6 days (Pfützner & Sachse, 1996) to 13.6 days (Punyapornwithaya et al., 2011). It should be noted, with the sampling carried out, the day of infection was an unknown number of days prior to the diagnosis. The measurement of the time from *M. bovis* infection to onset of clinical signs has been deemed challenging, as may depend on transmission route and infective dose (Calcutt et al., 2018). In agreement with this study, a Danish group (Petersen et al., 2018), reported 17/18 (94 %) M. *bovis* CM cows, had serum ELISA levels (BioX BioK 302 kit), above the recommended cut point, at the day of disease onset, whereas Australian work (Hazelton et al., 2018) found 15/16 (94%) *M. bovis* CM cows were serum ELISA positive (BioX K 302 kit) 7-13 days after the individual diagnosis of each cow. The findings of this study are consistent with the limited international work, and confirms the time since potential infection, needs to be considered when serum ELISA results are interpreted (Hazelton et al., 2018).

In contrast to this, other work in naturally infected animals has suggested that clinical disease has not always been followed by an increase of serum antibodies (Le Grand et al., 2002; Maunsell et al., 2011; Szacawa et al., 2016) and that there can be marked between-cow variability in antibody response, potentially due to pathogen or host factors (Petersen et al., 2018). Nevertheless, the antibody response in this outbreak suggests firstly, clinically infected cows were seropositive from disease onset, or shortly after disease onset, which secondly, infers cows, due to time to seroconvert, had been infected one to two weeks prior to becoming clinical and thirdly, there is a rapid rise in serum antibodies in the early phase of CM. For the veterinarian investigating an unexpected number of ATF CM cases, given the time interval for farmer diagnosis, first treatment period which is 3-5 days, potential milk WHP before the cow is cleared or retreated, she/he could expect a qPCR milk test detect to be accompanied by a positive serum ELISA test result.

5.3.2 Longevity of anti-M. bovis Antibodies

The smoothed function suggested that while the maximum mean serum SP% value (263) was reached at day 57 post CM diagnosis, it remained relatively constant until approximately 100 days when observations stopped, due to depopulation. While there are also gaps in the literature in the understanding of antibody longevity (Hazelton et al., 2018; Vähänikkilä et al., 2019), early experimental work showed IgA and IgG remained raised for 57 days (Bennett & Jasper, 1980), while a two-year study in 19 small infected Finnish farms (Vähänikkilä et al., 2019) showed, using serum MilA

ELISA, antibodies persisted on all farms, for at least 1.5 years. The infection was deemed resolved on six out of the 19 farms. In contrast Petersen et al. (2016) found, using the BioX ELISA, serum antibody levels declined within two months of clinical disease. BioX ELISA has been found to have a much lower sensitivity than MilA ELISA (Petersen et al., 2016; Wawegama et al., 2016). Pertinent to NZ, recent Danish work with the IDVet screen (ID screen® mycoplasma bovis indirect ELISA) test with a cut off value of SP% = 60 (Petersen et al., 2020) tested 12 herds with a range of current infection, *M. bovis* infection within the last five years (determined by persistence of serum and milk ELISA antibodies) and no infection. They found the five farms which had only had historic infection (mastitis and arthritis) in the last five years, had high numbers of cows (29/30, 28/30 and 23/30) seropositive. Further work is needed on the longevity antibody response to *M. bovis* infection.

5.3.3 Incidence of Clinical Mastitis

For Farms 1 (1050 cows) and Farm 2 (1000 cows), the cumulative incidence of *M. bovis* CM (0.046 and 0.042 respectively) and the incidence rate of first cases of *M. bovis* CM per 100-cow-months (0.95 and 0.87 respectively) were similar. There is, however, a dearth of international data on incidence rates during an outbreak of *M. bovis* CM, and the unit of measurement for incidence rates varies (Aebi et al., 2015; Punyapornwithaya et al., 2011). While an Italian (Radaelli et al., 2011) outbreak reported 45/122 (37%) cows developed CM, other work (Pfützner & Sachse, 1996) noted usually >20% of cows are affected with *M. bovis* mastitis in an outbreak. In a Finnish study (Vähänikkilä et al., 2019) only single cases of *M. bovis* CM occurred on 8/19 infected farms during the study period. The reported cumulative incidences in the NO outbreak, which were most likely under-reported as not all CM cows were sampled and the outbreak was only measured for part of the season, were similar to a large housed herd outbreak where <5% of cows developed *M. bovis* CM (Al-Abdullah & Fadl, 2006).

There are numerous opportunities for close contact in intensive pasture-based farming. During the spring of 2018, late pregnant cows and heifers on Farm 1 and 2 were highly stocked, often only being offered 15-20 sq m^2/day, as grass made up only about 40-50% of the 11kgDM springer diet. These cows would have been in close daily contact. In-shed feeding systems for meal/barley (Farm 1, a rotary had individual feeding bins, and Farm 2, a herring-bone had a continuous feeding tray) would have facilitated close cow-cow head contact during milking. Other management practices on these farms, common in this area of NZ, where there would be close cow-cow head contact include: firstly, the winter grazing management practice of strip grazing brassica crops i.e. kale or the long face of a high tonnage fodder beet crop with baleage/straw offered in feeders; secondly, strip grazing of fodder beet on the dairy platform in spring to finish a winter crop and/or in autumn to transition cows to fodder

beet for winter grazing; and thirdly to a lesser extent, in this area, the use of high tonnage summer crops i.e. summer turnips, where cows are offered small areas/day. Given the milking procedure is relatively similar regardless of farming systems, it could be proposed that our farming system, while more extensive than the housed total mixed ration (TMR) cow, offers numerous opportunities for transmission of the bacteria, resulting in adequate exposure time for the incidence and incidence rates to be similar, assuming other risk factors are similar. Further studies on the incidence rate of *M. bovis* infection in pastoral farming systems could better inform control programmes.

5.3.4 Age Incidence of M. bovis CM Cows

A large Saudi Arabian study (Al-Abdullah & Fadl, 2006) and Italian study (Radaelli et al., 2011) noted first-lactation heifers appeared at higher risk of clinical mycoplasma disease, including *M. bovis* CM. Clinical mycoplasma disease during the postpartum period, presumably mirroring the immunosuppression and stress of calving, is common (Parker, House, Hazelton, Bosward, Morton, et al., 2017). The age or parity distribution of *M. bovis* CM cases is not really elucidated in the literature. The small data set in this case study**,** suggests that on Farm 1, the heifers were under-represented, and the seven-year-old cows on Farm 1, and six-year-old cows on Farm 2 were over-represented. Possible reasons for this uneven distribution of cases amongst different age groups might include previous mastitis history in the older cows, or bias in selection of cases to sample by farm staff. There was also only a small number of six-year-old cows (13) on Farm 2. However, risk factors for *M. bovis* CM were not fully investigated or modelled in this study. The seasonal incidence, by age, of all CM cases on Farm 1 and 2, (Fig. 4-10), as compared to the seasonal incidence for 2018 season for CM herds recorded in the LIC database, which represented over 196,000 cows, from < 2.5 million cows in recorded herds, followed the same trend of increasing with age, except for the heifer incidence on Farm 2, which was higher than 3- and 4-year olds. High seasonal incidence of CM, by age, compared to LIC age data, supports the chronic history of CM on these farms.

However, data from the 'Age group prevalence of *M. bovis* serum ELISA' (Table 4-5) suggests that heifers (two-year olds) were as likely to seroconvert as any other age group. This under-representation of heifers with *M. bovis* CM heifers was unexpected as it might be expected seroconversion would follow clinical disease. While all the heifers across the farms were Teatsealed during late winter, it could be argued that Teatseal would offer better protection against *S. uberis,* than *M. bovis* because transmission of *M. bovis* can be by both direct and indirect routes. However, it is possible the heifers, hypothesized to be the first age group to be exposed to the disease agent, became colonised or infected to the point where they seroconverted, then antibody levels dropped off, but the immune

system developed a memory response where the early exposure acted like a vaccine. Hence during the peri-calving transition period the heifers were less likely to manifest clinical disease. Alternatively, seroconversion during mammogenesis/lactogenesis may afford some protective mechanism against expression of clinical disease. This area of *M. bovis* clinical disease needs further investigation.

5.3.5 Pattern of the CM Outbreak

A distinguishing initial clinical feature of the CM outbreaks on Farms 1 and 2 was the unusually high proportion of ATF cases, compared to a large NZ study which examined the effect of treatment on CM cases, where 21.9% and 25.1% ATF were reported in the treatment and control group respectively (McDougall et al., 2009). The stacked bar plots (Figs. 4-8 and 4-9) suggests there was a pattern to the *M. bovis* outbreak. Farm 1 had *M. bovis* CM diagnosed in week 3-4 from PSC, whereas Farm 2 was around three weeks later, in week 6-7 from PSC. No new cases of *M. bovis* CM were detected on Farm 1 from week 17 PSC (depopulation from week 24), even though samples of mastitic milk were still delivered to the clinic (Table 4-16) for processing. This compared to Farm 2, which had a higher peak of *M. bovis* CM cases around week 10 from PSC, and experienced *M. bovis* cases through to around week 21, with depopulation from week 23.

Even though the experience of working with ATF cows on Farm 1, later diagnosed with *M. bovis* CM, alerted Farm 2 when ATF cows emerged, the treatment success of early spring CM cows on Farm 2, would add support to the belief that the start of Farm 2's outbreak being some four weeks after Farm 1**.** However, as many CM cases were not sampled, it is not possible to make an inference about the causes of CM in these cows or the pathogens involved. The Programme deemed Farm 3 herd-level serology positive based on individual ELISA results from cow serum collected on 24.10.2018, some 8-9 weeks after Farm 1. On Farm 3 only one mastitic cow was tentatively diagnosed with *M. bovis* CM (27.12.2018).

There are three potential reasons for this difference between farms. Firstly, fewer infected heifers arrived onto Farm 3 from grazing. This is plausible as from discussion with farm managers, Farm 3 heifers were grazed on a different part of Farm 7, and only had limited contact with the potential *M. bovis* infected stock on that property. Secondly, Farm 3 heifers may have only become infected later in spring, when the final groups of heifers from the three farms were mixed on Farm 6 in late-August/early-September, for ease of management. Thirdly, differences in farm management practices e.g. higher intake of feed during the transition period on Farm 3, may have altered the transmission of *M. bovis.*

5.3.6 Subclinical IMI (M. bovis IMI)

Studies on the prevalence of subclinical IMI due to *M. bovis* are limited, as prior to the widespread use of PCR testing in recent years, emphasis was on culturing CM cases. The AP of subclinical IMI on Farm 1 in this outbreak was 0.75%, which is consistent with recent Australian work (Hazelton et al., 2020), where four herds, after outbreaks of *M. bovis* CM, reported low APs of subclinical IMI, in the main milking herds of 0.2%, 0.0%, 0.1% and 0.0%. Estonian work has reported different within-herd prevalences of *M. bovis* IMI. Timonen et al. (2017) reported a much higher prevalence of 17.2% in one herd but more recently (Timonen et al., 2020) based on CMS over a six-month period, showed a range of 12.3% down to 0.4%, with one farm having 3.4%, 0.4% and 0.4% respectively over the three tests.

The measurement of the AP of *M. bovis* subclinical IMI in milk is challenging as milk qPCR is epidemiologically insensitive. While *M. bovis* CM cows can be prolific shedders (>10^6 cfu/mL milk) (Biddle et al., 2003), well above the PCR limit of detection for *M. bovis* in milk of ~10^2 cfu/mL (Clothier et al., 2010; Rossetti et al., 2010; Parker et al., 2017), intermittent shedding into milk, is also a known feature of *M. bovis* mastitis, and may even fluctuate in one-week cycles (Wilson et al., 2009). These challenges suggest that any value of the AP of *M. bovis* subclinical IMI is inevitably going to underestimate true prevalence perhaps even in a major fashion.

5.3.7 Calf Surveillance Testing

Unlike Farms 1 and 2 where on-farm tested calves returned positive qPCR nasal swabs and also positive serum ELISA and qPCR tonsillar swabs collected at slaughter, all tested Farm 3 calves' (Table 4-15), tested negative on-farm qPCR nasal swabs and also serum ELISA and qPCR tonsillar swabs at slaughter.

The nose, like the ear, eye, prepuce and vulvovaginal tract, is an accessible mucosal site where *M. bovis* can colonise and be shed from by infected animals, irrespective of whether they are showing clinical disease or not (Hazelton et al., 2018; Jasper & Al Aubaidi, 1974; Punyapornwithaya et al., 2010). In herds with *M. bovis* clinical disease outbreaks, the prevalence of *M. bovis* in calf nasal swabs is often up to 100%, and low in herds that are known to have *M. bovis* but which have not experienced a disease outbreak (Bennett & Jasper, 1977b; Brown et al., 1998; Maunsell & Donovan, 2009; Vähänikkilä et al., 2019). Therefore, the low prevalence of qPCR positive calf nasal swabs on Farm 3 calf data suggests that transmission of *M. bovis* to calves on Farm 3 was low, including directly from the dam (from which, in NZ, the calf is usually separated within 24 hours), from unpasteurised colostrum/red milk fed to Farm 3 calves (Maunsell & Donovan, 2009) and from the respiratory

secretions of other calves (Nicholas, 2011). In contrast on Farms and 1 and 2 it is probable that calf infection occurred via any and/or all these routes.

5.4 Quarter-level Investigations

5.4.1 Other Pathogens Present in Milk of M. bovis CM Cows

The QMS from 50 glands from Farm 1 (from 41 *M. bovis* CM cows) and 47 glands from Farm 2 (from 37 *M. bovis* CM cows) (Table 4-17) were also analyzed using a DNA Mastitis Pathogen multiplex qPCR panel for common Gram positive mastitis pathogens in NZ, namely *S. aureus, S. uberis, S. dysgalactiae and S. agalactiae.* Farms 1 and 2 had a similar percentage of glands where only *M. bovis* was detected 32/50 (64%) and 29/42 (69%) respectively. In the remaining glands where one other pathogen was detected, *S. uberis* was the most common bacterial species detected. *S. uberis* is the most common cause of CM in NZ and is especially predominant in spring (McDougall et al 2007). Mastitis is considered to be caused by a single pathogen (Keane et al., 2013), with the identification of three or more species in the same sample an indication that a sample is contaminated (Schukken et al 2013). Two pathogens in the same sample is rare. McDougall et al. (2007) cultured two distinct bacterial species from only 7.6% of 1400 glands of cows with CM but this is thought to indicate co-infection.

There are very few studies looking at which pathogens are present in BTM alongside *M. bov*is in herds with udder infections (Fox et al., 2003; Miranda-Morales et al., 2008). There are equally few examining which pathogens are found with *M. bovis* CM at either the cow or the gland level. Pothmann et al. (2015) took QMS from 10 cows that had been identified as being positive for *M. bovis* on a CMS and identified *S. uberis* in one quarter from two cows, CNS from two quarters of one cow, and a non-uberis streptococci from one quarter from one cow. In contrast Radaelli et al. (2011) did not culture any other pathogens from the milk of 32 cows with *M. bovis* CM.

The significance of co-infection with *M. bovis* in the mammary gland is not well defined, as historically gland-level work has been done by culture, with well-known difficulties (Boonyayatra et al., 2010). While the gold standard in mastitis diagnosis is by milk culture of a bacterial pathogen from an infected cow (Oliver et al., 2004), the detection of a mixed infection of two or more mastitis pathogens is more common if PCR is used rather than culture (Keane et al., 2013). The mechanics, and strengths and weaknesses of molecular diagnostic tests over culture, including bias, for the detection of mastitis pathogens are well reported in the literature (El-Sayed et al., 2017; Gillespie & Oliver, 2005; Keane et

al., 2013). However, as qPCR does not distinguish between dead and living bacterial DNA, the other pathogens may also be DNA remnants from previous infections.

For this study, consideration must also be given to two other variables. Firstly, the freezing of samples, for some (estimate a third) of the samples delivered to the clinic by the farmers, the samples were frozen and secondly performance of the DNA Multiplex panel used to identify the non-*M. bovis* pathogens.

The literature is not consistent about the impact of freezing of milk samples on the recovery of mastitis pathogens, either by culture or qPCR, but it could be assumed to have only minor impacts (Paradis et al., 2012). There is sufficient evidence (Murdough et al., 1996; Schukken et al., 1989) to show that freezing and storage at -20°C, does not affect the viability of *S. aureus* on culture. Recovery of *S. aureus* (Bürki et al., 2015) may even be higher in frozen milk (Godden et al., 2002). For *S. aureus*, the DNA Multiplex qPCR panel used had a diagnostic sensitivity of 95.0% (95% CI: 75.1 to 99.8%) and specificity of 92.5% (95% CI: 79.6 to 98.4%), and for *S. uberis* a diagnostic sensitivity of 77.8% (95% CI: 40.0 to 97.2%) and specificity of 81.3% (95% CI: 67.4 to 91) (Gribbles Veterinary, personal communication, 21.4.2021). These figures are consistent with other work (Steele, 2015), given that Multiplex qPCR is less sensitive than a simplex PCR panel (El-Sayed et al., 2017).

The behaviour of *M. bovis* as a pathogen, in the mammary gland is apparently very different from its' behaviour as a respiratory pathogen where it is almost always found as a part of a pathogen complex (Bürki et al., 2015). The reason for this is not clear but some potential reasons include the lung is under constant challenge with each inspiration, while the mammary gland is under periodic challenge. The mammary gland in the lactating cow is flushed at milking once or twice a day, there are standard hygiene measures associated with milking routine and finally, there are innate and nonspecific immunity within the gland that makes pathogen establishment in the mammary difficult (Sordillo & Streicher, 2002). Further investigation into co-infection and the risks of co-infection in *M. bovis* CM is required.

5.4.2 Clinical Aspects of the North Otago M. bovis CM Outbreak

The NO outbreak of *M. bovis* CM, which occurred in the stressful postpartum to peak lactation period, had numerous similarities to the limited number of reported outbreaks of *M. bovis* CM internationally, where there were ATF CM cows, with multiple quarter involvement in cows that had no other clinical signs of disease (Parker, House, Hazelton, Bosward, Morton, et al., 2017; Pfützner & Sachse, 1996; Radaelli et al., 2011). There were two notable differences though. Firstly, this enterprise outbreak of

M. bovis CM was on large (1000 cows) pasture-based, seasonal calving farms, and secondly, the outbreak could only be measured for a limited time i.e. into January 2019, when the herds were slaughtered. While a herd-level diagnosis of *M. bovis*, by BTM qPCR, had already been made on Farms 1 and 2, ATF to antibiotic treatment for CM was the initial standout clinical feature that alerted the farmers, and subsequently the veterinarian to a potential *M. bovis* CM outbreak.

While cows may develop *M. bovis* CM in one, two, three or all four quarters (Byrne et al., 1998; Maunsell et al., 2011; Pfützner & Sachse, 1996), in this outbreak, a high number (Farm 1 87.5% and Farm 2 90%) of cows were first diagnosed (which were then QMS *M. bovis* qPCR detected) with a single quarter CM, which progressed to a second quarter in about two-thirds of the cows (Farm 1 67% and Farm 2 71%). A high proportion of cows (Farm 1 82.1% and Farm 2 74.1%) developed the second quarter mastitis on the same side of the udder as the first. This pattern has been reported in both experimentally and naturally infected cows (Byrne et al., 1998; Jasper & Al Aubaidi, 1974). Spread to a third quarter, in this outbreak (Farm 1 15.2% and Farm 2 28.5%) may well be underreported, as on-farm management decisions were made about ceasing treatment of ATF cows. Of note, the *M. bovis* CM cows remained generally healthy without systemic signs, but infected quarters became agalactic (or light quarter) (Farm 1 37.5% and Farm 2 41.2%). These findings are also characteristic of *M. bovis* CM (Al-Abdullah & Fadl, 2006; Jain et al., 1969; Pfützner & Sachse, 1996; Pothmann et al., 2015).

In the first instance the appearance of the mastitis, on Farm 1 and 2 was reported by the farmers as surprisingly unspectacular. Initially, the single gland mastitis was a slightly discoloured white colour, odourless and strippable. The mammary secretion of some *M. bovis* CM cows had a mild cheesy consistency. While this parallels the description of the experimentally infected cow (Jain et al., 1969), by the end of the first course of treatment, the milk usually had a discoloured brown appearance, and had not responded to treatment.

There has only been one short published review of *M. bovis* clinical disease in NZ, the index case for this national outbreak in July 2017, from South Canterbury (Hay, 2018). Unlike this NO case study where only CM was diagnosed, the South Canterbury case farm had dry cows with CM in all four quarters, abortions, and also high numbers of lactating cows which presented with non-responsive four quarter mastitis, with hard and painful udders. There were also cows with fetlock arthritis without concurrent mastitis, and calves that showed a type of dysmaturity syndrome.

5.4.3 Antimicrobial Therapy for M. bovis CM

An outbreak of CM might be the first clinical sign that a farmer with potential *M. bovis* CM sees. On Farms 1 and 2, there was high use of intramammary antibiotics for treatment of CM cows, especially the first infected gland with *M. bovis*. The number of ATF *M. bovis* CM cows, Farm 1 37/48 (77%) and Farm 2 30/42 (71%), together with the high use of intramammary antibiotics is in agreement with work describing the control of *M. bovis*-associated diseases, especially mastitis. Antimicrobial therapy is largely considered unrewarding, as disease caused *by M.* bovis infection is often refractory to treatment (Ayling et al., 2014; Calcutt et al., 2018). However, β-lactam antibiotics (parental Mamyzin, intramammaries of Intracillin, Clavulox, Penclox, and Mastiplan) were the predominant family of antibiotics used especially early in this outbreak, with parental Tylan and the intramammaries of Mastlaone and Albiotic, used to a lesser degree. As *M. bovis* lacks a cell wall they are inherently not susceptible to penicillins, their derivatives, and cephalosporins (Maunsell et al., 2011; Nicholas et al., 2016).

Three challenges face the NZ clinician should they need to prescribe antimicrobials against *M. bovis* CM. Firstly, international work reports an increasing in vitro resistance, or developing resistance, by *M. bovis*, against several, if not all common classes of antibiotics (Ayling et al., 2000; Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016) This includes the tetracyclines, macrolides, fluroquinolones and spectinomycin in the aminoglycoside class (Calcutt et al., 2018; Pothmann et al., 2015). Secondly, many of the drugs used overseas are not commercially registered in NZ. The Veterinary Council of New Zealand (Te Kaunihera Rata Kararehe o Aoteoroa) - Code of Professional Conduct for Veterinarians: Veterinary Medicines which came into force on 1.1.2020, now requires veterinarians to have pharmacological justification to prescribe macrolides, fluoroquinolones and third and fourth generation cephalosporins (Veterinary Council of New Zealand, 2020). The macrolides include tylosin (Tylan), and oleandomycin, which is an active in Mastalone, the only oxytetracyline active intramammary available in NZ. Our sole lincosamide intramammary (with lincomycin as the active, Albiotic) currently does not have a registration for extended use beyond three 12-hourly treatments. The NZ clinician and farmer have very limited, if any, effective antimicrobial therapy against *M. bovis* CM. Thirdly, the copious amounts of exudates present and extensive pathology present on PM of the 14 known *M. bovis* CM would question the ability of antimicrobial intramammary infusions to disperse in the mammary gland and be of therapeutic value.

In this outbreak, Farms 1 and 2 had been deemed herd *M. bovis* positive, by BTM PCR, before the farmers became aware of a high number of ATFs, especially on Farm 1. As with any mastitis investigation the clinician needs to be aware of the differential diagnoses when presented with clinical

disease. While the diagnostic sensitivity of QMS *M. bovis* qPCR is considered low (Sawford, 2019), a very high number of QMS from CM mastitis cows (Table 4-8) were *M. bovis* PCR positive. While NZ is in the process of eradicating *M. bovis* and therefore it would be highly unlikely for a clinician to be faced with a case of *M. bovis* CM, in the face of an outbreak of CM accompanied by ATF collection of milk samples from as many cows with ATF as possible and testing with a Multiplex PCR panel that includes *M. bovis* is recommended.

5.5 Part 2 Agreement Study

Agreement between the serum ELISA, an immunodiagnostic assay, and palatine tonsil qPCR, a molecular diagnostic technique, in a multi-farm outbreak, has not been reported in the literature. Two different populations were considered in the agreement study which compared how well *M. bovis* serum ELISA (SP%) agreed with the palatine tonsil qPCR swab. Firstly *M. bovis* CM cows from Farm 1 and 2 were sampled on-farm and then at slaughter, and secondly, non-clinical cows from Farms 1 and 2 were sampled at slaughter only. The higher observed agreement was for *M. bovis* CM cows sampled on-farm, as indicated with the Gwet's agreement coefficient result a 'near perfect' judgement (agreement).

Studies have revealed a low prevalence of colonisation and shedding of *M. bovis* from accessible mucosal surfaces (nose, eye, ear and vagina) in naturally infected *M. bovis* CM cows, using PCR and culture techniques (Hazelton et al., 2020; Punyapornwithaya et al., 2010). The detection of a subclinically infected animal also poses challenges as there is both no consistently infected body site that can be sampled, or in the case of detection of SCM, there is intermittent shedding of *M. bovis* (Biddle et al., 2003; Gonzalez et al., 1992). In oral inoculation studies, the initial site of colonisation of *M. bovis* appears to be in the URT (Bennett and Jasper 1977, Brys and Pfutzner 1989.) Fourteen days after oral inoculation of calves, Maunsell et al. (2012) showed the tonsils were a major site of *M. bovis* colonisation in the URT. While, there is little published information about tonsil colonisation in naturally exposed calves/cattle (Buckle et al., 2020), the paired palatine tonsils, which are located at the junction of the oropharynx and nasopharynx, are in an ideal position to sample antigens which enter via the oral or nasal cavities. The reticular epithelium of the tonsil is known to contain key immune cells, and potentially M-cell-like cells. These cells enable uptake and processing of an antigen and importantly initiate an immune response (Palmer et al., 2009). It would follow then, that palatine tonsil colonisation may play an important role in the pathogenesis of *M. bovis* disease.

On-farm tonsil swabbing of CM cases, by clinical veterinarians, gave much higher agreement with ELISA test results, up to 30% greater using Gwet AC1 statistical test on Farm 1, compared to tonsil swabbing of CM cows at slaughter. There was a moderate agreement of non-clinical cows at slaughter on Farms 1 and 2. On-farm tonsil swabbing, a straight forward procedure in dairy stock, may offer a valuable diagnostic tool for the detection of *M. bovis* DNA and tentative diagnosis of *M. bovis* in a dairy herd, especially in non-lactating stock or young stock replacements. Similarly, it may be of value in countries where *M. bovis* is endemic.

The prevalence of tonsil qPCR positive cows from swab collection at slaughter may vary for several reasons but is potentially influenced by sample collection and handling methods. The sampling

techniques used on-farm and at slaughter in this study varied. A guarded swab was used on farm, with the gag being disinfected and dried between use. The guarded swab was used to reach the tonsillar crypt and prevent contamination between cows. In contrast, after slaughter the cows' heads are high pressure washed, on the PM rail, in order to remove ingesta contamination (and blood) prior to routine PM inspection (Ministry for Primary Industries, 2015). Tonsil swab sampling post high pressure hosing of the head may decrease the ability to collect *M*. *bovis* DNA on a swab. Additionally, personnel on PM examination rotate positions within a shift, possibly with different swabbing techniques, which could further decrease agreement between serological and tonsillar swab test results.

The agreement data set also allows comment on the proportion of the two populations that were palatine tonsil qPCR positive (detected). In the CM cows combined from both herds, on-farm 59/65 (90.8%) were detected, and at slaughter 47/65 (72.3%). The non-clinical slaughter saw Farm 1 39/82 (47.6%), Farm 2 113/226 (50%), and the prospective cohort study (from Farm 1) 40/100 (40%). From these data it appears that CM cows have higher tonsil colonisation than non-clinical cows. There may be direct and indirect transmission of *M. bovis* in an infected dairy herd during peak lactation and given that systemic hematogenous spread of *M. bovis* has been postulated (Biddle et al., 2005; Fox, 2012) there may also be udder – tonsil spread. This area of understanding needs further investigation, including the circulation and lymphatic draining of the palatine tonsil.

5.6 Part 3 Pathological Findings

The most consistent gross lesions in the udders of 14 known *M. bovis* CM cows, from Farm 1, at slaughter (55 quarters) were fibrosis, caseous necrosis and cystic dilation of the mammary parenchyma. While the volume and consistency of the exudate varied, lesions were seen in at least three quarters in ten of the cows. The index case of farmer-detected *M. bovis* CM on this was farm was 110 days prior to slaughter, so considering diagnosis date for these slaughtered cows, the lesions ranged in duration from around 50 days (cow #7, Tag Id.435) to around 105 days (cow #6, Tag Id. 1435). There has been limited description of gross pathology of naturally occurring cases of *M. bovis* CM.

Much of the description of gross pathology of *M. bovis* CM has been in experimentally induced cases (Bennett & Jasper, 1977a; González & Wilson, 2003; van der Molen & Grootenhuis, 1979), with lesions characterized as mild to severe fibrinosuppurative to caseonecrotic mastitis. While a large Saudi Arabian outbreak (Al-Abdullah & Fadl, 2006) only considers histopathological findings, a small Austrian study briefly reported the gross pathology and histopathology findings from one cow (Pothmann et

al., 2015). In an Italian outbreak, Radaelli et al. (2011) described the necropsy of three cows – two with mild, chronic, suppurative mastitis and galactophoritis, and the other a chronic, necrosuppurative and pyogranulomatous galactophoritis. Studies that have evaluated a correlation between lesion characterisation with the mastitis causing pathogen are scarce (Benites et al., 2002; Bianchi et al., 2019; Hazlett et al., 1984; Hussain et al., 2012).

Fibrosis, the most common lesion seen in these quarters, is part of the repair process in damaged mammary parenchyma and is the start of cicatrix formation (Benites et al., 2002). Fibrosis can commence during an inflammatory response or develop from cystic dilation. Cystic dilation is also a form of repair, where cysts are formed in dilated acini of the mammary gland (Jones & Hunt, 1983). These changes will impair milk production in affected cows and clinically, agalactic (light) quarters result. This is a noted finding in *M. bovis* CM cases (Calcutt et al., 2018; Radaelli et al., 2011) and was seen in this outbreak.

Histopathological findings in CM cows e.g. significantly lower alveolar diameter, number of alveoli and alveolar epithelial population (Hussain et al., 2012), findings of morphometric analysis of mastitic mammary tissue (Sordillo et al., 1989) and experimental findings of *M. bovis CM* cows such as chronically infected quarters consisting of alveolar involution and moderate to severe mononuclear infiltration, with an increase in interalveolar connective tissue (Bennett & Jasper, 1977a; van der Molen & Grootenhuis, 1979), all may be suggestive or representative of a mastitis pathogen but not pathognomonic. There are several cow-level factors that may determine histopathological findings including pregnancy status, time since calving, stage of lactation, and stage of inflammatory response whether acute or chronic (Helmboldt et al., 1953; Sordillo et al., 1989; Trinidad et al., 1990). Similarly, morphological characterisation is not pathogen specific. *S. aureus* may present as a suppurative, abscedative, lymphoplasmacytic, mixed, or pyogranulomatous mastitis (Bianchi et al., 2019).

Granulomatous inflammation was the most common morphologic pattern seen in histopathology in this outbreak (33%), followed by suppurative inflammation (27%). While this is a novel finding for *M. bovis* CM cases, and unlike other common mastitis pathogens, several pathogens can cause a granulomatous inflammation i.e. *Mycobacterium bovis*, *Prototheca zopfii, Nocardia* spp and uncommonly the chronic form of *S. aureus* (botryomycosis) (Bianchi et al., 2019; Corbellini et al., 2001). The 23 sections of granulomatous inflammation in this outbreak, were negative for AFO (under the Ziehl-Neelsen stain) which would help exclude bovine tuberculosis. This was a valid rule out, given Farm 7 was in the vicinity of a bovine tuberculosis infected farm. While not performed, Grocott's (Gomori) methenamine silver histological stain can be used to identify fungal organisms, including rule out of *P. zopfii,* an uncommon cause of mastitis in New Zealand. The presence of the Splendore

Hoeppli (asteroid bodies), often seen in the centre of pyogranulomatous inflammation, would incriminate other bacterial organisms, such as *S. aureus* as the mastitis-causing pathogen in cases of botryomycosis (Bianchi et al., 2019).

In this study, only 2/41 sections had a positive Gram stain, the Gram stain having poor sensitivity. Considering data from the Multiplex qPCR quarter sampling (Table 4-17), 5/12 quarters had *M. bovis* plus one other pathogen present. Of note though, for four *S. uberis* detections there was a range of inflammatory responses reported histologically. No routine diagnostic milk cultures were done in these CM cows. *Mycoplasma bovis* IHC studies showed 55% of quarters were immunoreactive for the *M. bovis* antigen, and 87% of these quarters were either granulomatous or pyogranulomatous, but only *M. bovis* antigen was tested for. Nevertheless, the pathological studies add additional diagnostic support to the likelihood of *M. bovis* being involved, as at least one of the pathogens, in these CM cows.

5.7 The Diagnosis

Epidemiological and clinical findings of *M. bovis* CM at the herd-, cow- and quarter-level have been reported and provide strong evidence of the involvement of *M. bovis* in the CM outbreak across a large-scale dairy operation in NO. The course of the outbreak on Farms 1 and 2 was dynamic, with Farm 2 lagging Farm 1 by three to four weeks. With herd depopulation the outbreak was only followed for a limited time. Key diagnostic findings include BTM PCR positive results, supported by positive BTM ELISA, which suggests exposure and infection within the herd; an unusually high incidence of ATF for cases of CM, from some of which *M. bovis* was detected; and individual CM cases were QMS *M. bovis* qPCR test positive, with associated serum ELISA positivity. Additionally, *M. bovis* was the sole pathogen found in around 70% of CM cows by a mastitis Multiplex qPCR. There was broad clinical presentation of poor response to antimicrobial therapy, where CM which started innocuously in one quarter, spread to multi-quarter mastitis with light quarters, where the infected cows remained systemically well. Further supportive diagnostic evidence was the gross lesions of fibrosis, caseous necrosis, and cystic dilation, together with the granulomatous and suppurative inflammatory patterns seen histologically and the high immunoreactivity in IHC for *M. bovis* antigen.

From these key findings, *M. bovis* was likely to have been one of potentially several pathogens that caused individual cases of CM apparent on Farms 1 and 2, and in many cases may have been the sole cause of CM cases.

5.8 Limitations

Several limitations of the methods of animal sampling and diagnostic tests used in this study have already been discussed. Some of these were due to the constraints of resources and the fact that the study was carried out on commercial herds during a stressful period for owners and staff because the *M. bovis* Programme was underway (New Zealand Government, 2018).

Any on-farm and slaughter surveillance and sampling of CM cases have both practical and potential logistical difficulties. The timing of sampling during milking during an on-farm surveillance visit for individual cow testing may have influenced the test results from ATF cows. These cows were more likely to be a *M. bovis* CM case and seropositive, and more likely to be in the red mob and milked at the end of milking. Therefore, they may not have been representative of other CM cows in the herds. The selection of CM cows sampled and examined by the veterinarian (author) were farmer-selected, farmer-collected and reported with potential non-random bias in selection and an under-representation of *M. bovis* CM cases, as not all CM cases were sampled. The accuracy and completeness of farmer-recorded CM treatment could not be verified, especially the quarters treated, and hence, results from analysis of these records were also biased to some extent. These problems with data quality may have been due to farmer fatigue, failing interest with cows being depopulated, with the likely outcome an under-reporting of CM cases. There were non-standardised on-farm decisions made about treatment of CM cows throughout the outbreak, as all cows were destined for slaughter. The definition of a *M. bovis* CM case was widened to allow for this. Additionally, the outbreak was only followed for a limited time, and the full extent of this outbreak, if it had more time to evolve could not be determined.

To ensure cows were correctly identified to be included in this study to the correct farm, each cow's RFID tag was linked to her LID and then management tag. Incorrect tag details excluded some cows from the study. During the outbreak, only three or four on-farm clinical visits were made to sample and examine CM cows. Therefore, the number of cases in some clinical data sets were small e.g. assessment of agalactic quarters, *M. bovis* CM by age (years), co-infection with *M. bovis* CM. This may lead to over-interpretation of findings because of random sampling variation. On sampling the mammary glands at the freezing works, only one representative sample per quarter was taken for analysis, whereas different parts of the quarter with possibly different predominant lesions were not sampled. For cost purposes, IHC reactivity for *M. bovis* only, and no other mastitis pathogens was carried out. The addition of antigen from other pathogens would have provided supportive evidence for the co-infection of *M. bovis* CM.

The limitations of diagnostic tests are largely measured by their sensitivity and specificity (Table 3-4) in a population with given characteristics and stages of infection. These measures may be inexact and vary throughout the course of natural infection. In some situations, the population in which they were validated for, may not have been detailed which did not aid the interpretation of tests in the study herds. In outbreaks as in this study, these diagnostic tests are used firstly, to make inferences about cows and the herds they are located in, secondly, to communicate with interested parties and thirdly, to make decisions about disease control in a national herd. The *M. bovis* qPCR test has poor diagnostic sensitivity (Ministry for Primary Industries, 2019b; Sawford, 2019); the bacteria can be shed intermittently in cows with IMI (Biddle et al., 2003) and the spectrum of the course of an *M. bovis* infection in an individual cow, can range from subclinical and transitory to clinical and persistent, with a range of different organ systems even involved. These unique biological features of *M. bovis* challenge the field diagnosis of this disease.

5.9 Future Research

In 2018, *M. bovis* was listed as an Unwanted Organism in NZ, under the Biosecurity Act 1993, with an eradication objective (Ministry for Primary Industries, 2019a). This changes the environment for future research in NZ, as infected herds are depopulated. However, there are a number of potential future studies, which would add to the results of this study. These include: quantifying the transmission rate of *M. bovis* in a pasture-based dairy system; the modelling of risk factors for large-scale pastoral-based systems; data on QMS *in vitro* culture antibiotic sensitivity of *M. bovis,* (in NZ), to equip the clinician with more informed clinical treatment options, prior to final herd slaughter; further Multiplex DNA sampling of *M. bovis* CM cases to investigate how commonly *M. bovis* acts as the sole pathogen in CM cases in contrast to the pathogen's behavior in the respiratory complex; and finally, the role and significance of hematogenous spread of *M. bovis* in *M. bovis* CM cases, given they can also develop pneumonia and *M. bovis* arthritis (Punyapornwithaya et al., 2011).

Chapter 6 Appendices

6.1 Appendices for Chapter 3 Materials and Methods

6.1.1 Document 1 Antimicrobial Therapy Used for Mastitis Treatments

Parental Antimicrobial Therapy

Mamyzin: 10g and 5g vials of penethamate hydriodide [Mamyzin, Boehringer Ingelheim (NZ) Ltd., Auckland, NZ]. A 10g vial, reconstituted with the water for injection, is injected subcutaneously (s.c) or intramuscularly (i.m) in the anterior half of the neck on day one, followed by a 5g vial, reconstituted, on day two. Milk WHT: 60 hours after the last treatment of a 10g plus 5g dosage regime. Meat WHT: 7 days.

Tylan 200 injection: 200 mg/ml Tylosin (as tylosin base) in a 50% propylene glycol solution [Tylan 200 Injection, Elanco Animal Health, Auckland, NZ]. 5-10mg/kg body weight i.m in neck muscle daily, not to exceed 5 days of treatment e.g. 25ml i.m daily for 3 days. Milk WHT: 72 hours after last treatment. Meat WHT: 21 days.

Intramammary Infusions/Syringes

Intracillin 1000 Milking Cow: 1,000,000 i.u procaine penicllin [Intracillin 1000 Milking Cow, Virbac NZ Ltd., Hamilton, NZ]. An extended treatment of up to 6 syringes, with 1 syringe being infused into each infected quarter immediately following each successive milking i.e. every 12 hours. Milk WHT: during treatment and not less than 8 milkings or approximately 96 hours following the last treatment. Meat WHP: 10 days.

Clavulox L.C.: a combination of 200mg amoxycillin, 50mg clavulanic acid (as potassium clavulanate) and 10mg prednisolone in a 3g mineral-oil base for intramammary infusion. [Clavulox L.C., Zoetis NZ Ltd., Auckland, NZ]. A course of up to 5 syringes per infected quarter, with one syringe being infused every 12 hours. Milk WHT: (5 syringes) during treatment and not less than 8 milkings or approximately 96 hours following the last treatment. Meat WHP: 7 days.

Albiotic: a combination of 330mg lincomycin (as lincomycin hrdrochloride) and 100mg of neomycin (as neomycin sulphate) in each 10ml dose. [Albiotic, Agrihealth NZ Ltd., Auckland, NZ]. For twice daily milking: treatment may be repeated at 12 hour intervals, up to a total of 3 doses. Milk WHT: during treatment and not less than 5 milkings or approximately 60 hours following the last treatment. Meat WHT: 10 days.

Penclox 1200: a combination of 1000mg procaine penicillin and 200mg cloxacillin [Penclox 1200, Virbac NZ Ltd]. An extended treatment of 4, 5 or 6 syringes at 24-hour intervals may be given. Milk WHP: up to 6 treatments, when milking twice-a-day, during treatment and for not less than 9 milkings or approximately 108 hours following the last treatment. Meat WHP: 10 days.

Mastiplan: a combination of 300mg cephapirin (as sodium salt) and 20mg prednisolone [Mastiplan, MSD Animal Health, Upper Hutt, NZ]. One syringe is infused into each affected quarter, immediately after milking, at 12 hour intervals for four consecutive milkings. Milk WHT: not less than 10 milkings or 120 hours following the last treatment. Meat WHP: 3 days.

Mastalone: a combination of 200mg oxytetracycline HCl, 100mg oleandomycin base (as phosphate), 100 mg neomycin (as sulphate), and 5mg prednisolone [Mastalone, Zoetis NZ Ltd]. One 10ml syringe should be infused into each affected quarter immediately after milking, repeated at 24 hour intervals for three days. Milk WHP: during treatment and for not less than 8 milkings or approximately 96 hours following the last treatment. Meat WHP: 30 days.

6.1.2 Document 2 Laboratory Analysis Methodologies (Mycoplasma bovis)

6.1.2.1 DNA Extraction AHL, Wallaceville

Purification of genomic DNA was done using QIAcube HT (Qiagen) using QIAamp 96 DNA QIAcube HT kit. The protocol "Purification of genomic DNA from tissue samples" described in the QIAamp 96 DNA QIAcube HT handbook (December 2013) was used, with minor modifications.

Briefly, each swab was re-suspended in 1 mL PBS and centrifuged at 10,000 Xg for 3 min. After removing the supernatant, 180uL of ATL buffer and 20uL of proteinase K solution were added into the pellet and mixed by vortexing. The samples were then incubated at 56°C overnight for cell lysis to complete. Following lysis, 100uL of VXL buffer was added and incubated at 65°C for further 10 min for inactivation. Three hundred microliters of sample was added to the S block and this was placed in the QIAcube for extraction with an elution volume of 100uL.

6.1.2.2 DNA Extraction Gribbles, Palmerston North

DNA was extracted from QMS and CMSs either using the Magmax CORE Nucleic acid purification extraction kit (ThermoFisher Scientific New Zealand Limited) automated on a KingFisher Flex instrument (Thermofischer Scientific, New Zealand) or manually using a Zymo ZR Fungal-bacterial DNA kit (Ngaio diagnostics, New Zealand).

6.1.2.3 Mycoplasma bovis qPCR Testing at AHL, Wallaceville

Real time PCR analysis to detect bacterial DNA of *M. bovis* was carried out using VetMAX™ *M. bovis,* (TaqVet™ *Mycoplasma bovis* Real-time PCR, France and formerly known as LSI VetMAX *Mycoplasma bovis* kit) a commercial quantitative PCR test kit (Thermo Fisher Scientific Inc, catalogue number MPB050, distributed through Life Technologies New Zealand Ltd).
Real time PCR analysis testing was carried out according to manufacturer's instructions (*https://www.thermofisher.com/order/catalog/product/MPBO50#/MPBO50)* where the detected target of VetMAX™ is polC gene.

In brief, the VetMAX™ *M. bovis* kit contains reagents for the detection in duplex of *M. bovis* and an internal control IPC. The kit is stored at -30°C to -10°C. The qPCR reaction volume is 25µL, which is made up of 20µL per analysis of the test kit component 3-Mix MPBO, thawed to between 2°C and 8°C, and then added to each well of the PCR plate, PCR strip or capillary used. For sample analysis, 5µL of

extracted DNA is added. The PCR plate, strips or capillaries are then covered with an adhesive plate cover or suitable caps and processed.

6.1.2.4 *Mycoplasma bovis qPCR Testing at Gribbles, Palmerston North*

DNA samples were tested using a commercial *M. bovis* real time PCR assay (*Mycoplasma bovis duo real time PCR kit)*, DNature diagnostics and research Ltd, NZ) on the Qiagen Rotor-Gene Q PCR platform according to manufacturer's instructions.

Within the Programme, qPCR samples were considered positive if both sample and internal positive control CT values were less than 45.

6.1.2.5 *Mycoplasma bovis-specific Antibodies Using Indirect ELISA*

Bulk tank milk samples and serum samples from cows were analysed for *M. bovis*-specific antibodies using the commercial ID.Vet ID Screen® ELISA (Innovative Diagnostics, Grabels, Montpellier, France and distributed by Q A Diagnostics Ltd, Albany, Auckland). This IgG ELISA was developed from a recombinant fragment of mycoplasma immunogenic lipase A (milA) (Wawegama et al., 2014). Analytical specificity and sensitivity for this test are 100% and 95.7% respectively (ID.vet, 2018).

In NZ, the ID.ELISA test result has been reported as an SP% (sample to positive) with a cut point of 60% or above as a positive test result for an individual animal and 30% or above for a BTM sample. These indirect ELISA tests were performed on the samples according to manufacturer's instructions (*https://www.id-vet.com/product/id-screen-mycoplasma-bovis-indirect/*) (ID.vet, 2018)

6.1.2.6 *Histopathology for M. bovis*

Following fixation in 10% neutral-buffered formalin, histologic sections of mammary tissue were prepared routinely and stained with haematoxylin and eosin (HE) by the Histopathology Laboratory, School of Veterinary Science, Massey University. In addition to routine HE staining, a Gram stain was performed on each section displaying granulomatous or suppurative inflammatory lesions. Ziehl-Neelsen stain was performed on sections with evidence of granulomatous inflammation.

Immunohistochemisty

Immunohistochemistry for detection of *M. bovis* antigen was done by the Animal Health Laboratory, University of Guelph, Canada. Automated IHC procedures were performed using a DAKO autostainer (DAKO Cytomation Inc., Mississaugua, Ontario, Canada). The primary antibody was a rabbit polyclonal

anti-*M. bovis* antiserum (supplied by Dr. Maureen K. Davidson, Purdue University, West Lafayatte, IN, USA) applied at a 1:300 dilution for 60 minutes at room temperature. Non-immune rabbit serum was substitute for the primary antibody in negative control slides. Antigen retrieval was an enzymatic method (proteinase K) run at room temperature for 12 minutes. Goat anti-mouse immunoglobulin conjugated to a horseradish peroxidase-labelled polymer (EnVision HRP, DAKO Cytomation Inc., Mississaugua, Ontario, Canada) was used as the secondary antibody, with a 30-minute incubation at room temperature.

Evaluation of immunoreactivity was performed by a qualitative method, indicating the presence (+) or absence (-) of immunoreactivity as previously reported (D. Adegboye et al., 1995). Based on previous immunohistochemical studies of *M. bovis* (D. Adegboye et al., 1995; D. S. Adegboye et al., 1995) a positive result was based on evidence of strong immunoreactivity within foci of necrosis or within the cytoplasm of intralesional neutrophils and macrophages. The evaluation was conducted by a board-certified pathologist from the Animal Health Laboratory, University of Guelph.

6.1.2.7 DNA Mastitis Pathogen Multiplex qPCR

Individual cow milk samples which were processed at Gribbles, were also tested for *S. aureus, S. uberis, S.dysgalactiae and S. agalacatiae* using a Mastitis multiplex real time PCR on the Qiagen Rotor-Gene Q PCR platform (Qiagen, Hilden, Germany). This assay was developed and validated at Gribbles. The *S. aureus* (Graber et al., 2007) and *S. agalactiae* (Gillespie & Oliver, 2005) assays were based on previously published primers and probes. Primers and probes for *S. uberis* and *S. dysgalactiae* were designed and validated in-house.

6.1.3 Document 3 Other Laboratory Analysis

6.1.3.1 *Gribbles – Trace Element and Day 3 Post Calving Blood Profiles*

Serum Calcium

Roche Diagnostics 05061482-190 run on Roche Cobas biochemistry analyser.

Calcium ions react with 5-nitro-5'-methyl-BAPTA (NM-BAPTA) under alkaline conditions to form a complex. This complex reacts in the second step with EDTA. The change in absorbance is directly proportional to the calcium concentration and is measured photometrically.

Serum Magnesium

Roche Diagnostics 06481647-190 run on Roche Cobas biochemistry analyser.

Magnesium ions form a purple-red complex with xylidyl blue in alkaline solution. The magnesium concentration is measured in terms of the decrease in absorbance of xylidyl blue at 505nm. EGTA contained in the buffer prevents calcium interference.

NEFA (Non-esterfied fatty acids)

Wako kit 279-75401 run on Roche Cobas biochemistry analyser.

Non-esterified fatty acids in serum, when treated with acyl-CoA synthetase (ACS) in the presence of adenosine triphosphate (ATP), magnesium ions and CoA, form the thiol esters of CoA known as acyl-CoA.

In the second reaction, acyl-CoA is oxidised by acyl-CoA oxidase (ACOD) to produce H2O2, which in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) to form a purple adduct measured at 550nm.

Ascorbate oxidase (AOD) is added to prevent ascorbic acid in sample reacting with H2O2.

Serum B12

Vitamin B12 is extracted from its protein complex by boiling at pH 4. A known amount of 57Co-B12 is then allowed to compete with the extracted B12 for binding to a limited amount of B12 - binding protein. Finally, bound and free portions of B12 are separated. Measurements of the amount of bound 57Co-B12 allows determination of the degree of radioisotopic dilution, and hence the amount of sample B12 present, by comparison to a standard curve (Green et al., 1974; Millar et al., 1984).

Serum Selenium and Copper

In-house procedure, based on Perkin Elmer Application Note: ICP-MS Nexion 2000: Determination of Trace Elements in Blood and Serum

Inductively Coupled Plasma Mass Spectroscopy or ICP-MS is an analytical technique used for elemental determinations. ICP-MS combines a high-temperature ICP source with a mass spectrometer. The ICP source converts the atoms of elements in the sample to ions. These ions are then separated and detected by the mass spectrometer.

6.1.3.2 *LIC BTM BVD Ag PCR and LIC BTM BVD Ab ELISA*

BTM BVD (Ag) PCR

BTM BVD PCR is tested by the Bovine Virus Diarrhea RNA Test Kit, VetMAX (TM) – Gold BVDV PI Detection Kit; Applied Biosystems, Life Technologies brand of Thermo Fisher Scientific Corporation. Carried out under manufacturer's instructions
https://www.thermofisher.com/order/catalog/product/4413938#/4413938

BTM BVD Ab ELISA

BTM BVD Ab ELISA is tested by Bovine Viral Diarrhoea Virus (BVDV) Antibody Test Kit, IDEXX BVDV Total Ab, IDEXX NZ. Carried out under manufacturer's instructions.

https://www.idexx.co.nz/en-nz/livestock/livestock-tests/ruminant-tests/idexx-bvdv-total-ab-test/

6.1.3.3 *Bovine Viral Diarrhoea Virus (BVDV) Individual Cow*

Individual cows were tested for BVDV Ag at Gribbles. The IDEXX BVD antigen/Serum Plus kit was used. These indirect ELISA tests were performed on the samples according to manufacturer's instructions.
https://www.idexx.co.nz/en-nz/livestock/livestock-tests/ruminant-tests/idexx-bvdv-agserum-plus-test/

Individual cows were tested for BVDV Ab ELISA at Gribbles using the BVDV Antibody Test Kit, IDEXX BVDV Total Ab, IDEXX NZ. These indirect ELISA tests were performed on the samples according to manufacturer's instructions. https://www.idexx.co.nz/en-nz/livestock/livestock-tests/ruminant-tests/idexx-bvdv-total-ab-test/

6.1.4 Document 4 Methodology of Prospective Cohort study

On Farm 1, 100 cows were blood tested for *M. bovis* serum ELISA on the 18.9.2018 surveillance visit 1. A second visit to blood sample these cows was scheduled for 10.1.2019. However, 13 of these cows had been slaughtered by then as 11/13 were *M. bovis* CM cases (three were slaughtered on 29.10.2018 on-farm, nine on 4.12.2018 and one on 17.12.2018.) To make this sample back to 100 cows, the herd was stratified by age, as this was considered the simplest from a descriptive epidemiology perspective, and seven to eight cows from each of eight age groups (two-year old to nine plus) were randomly selected for blood testing and other sampling.

On the 10.1.2019, 133 of the 140 cows selected for sampling were drafted during morning milking. They were blood sampled and their sera tested by *M. bovis* ELISA, and CMS (3 strips per quarter) for milk qPCR. From the original 100 cows, 82 cows had paired serum blood results (sampling dates 18.9.2018 and 10.1.2019). Thirteen had been slaughtered, two cows were not drafted/not found and there were three inconsistencies with tag identification/labels.

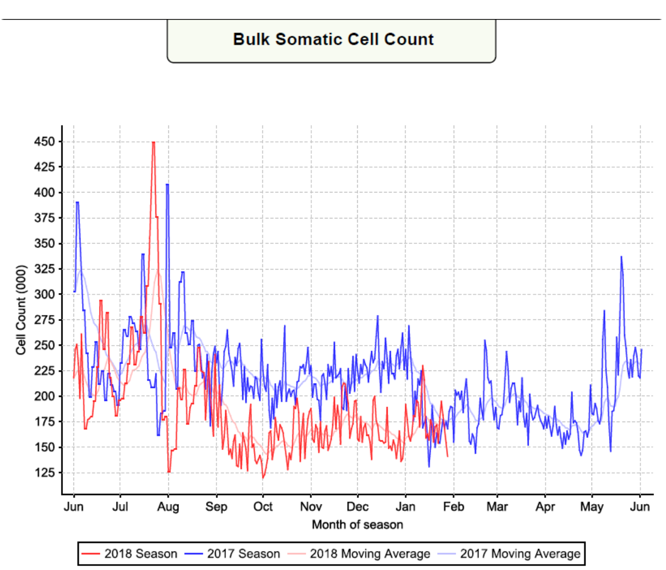
Farm 1 data to be used includes: seroconversion results from serum ELISA testing of 133 cows on 10.1.2019; and CMS from 133 cows sampled for milk qPCR, for determination of subclinical IMI prevalence. The 133 cows were slaughtered over two days at South Pacific Meats (SPM) Canterbury, with 100 qPCR tonsil swabs being taken on 25.1.2019. It was considered the 15-day time interval between serum ELISA testing on farm on the 10.1.2019, was too long to include this data set in the Agreement study (4.4). Current knowledge of serum antibody longevity and dynamics is insufficient to assume the serum ELISA antibody level would not change in 15 days, and thereby change the test outcome.

6.2 Appendices for Chapter 4 Results

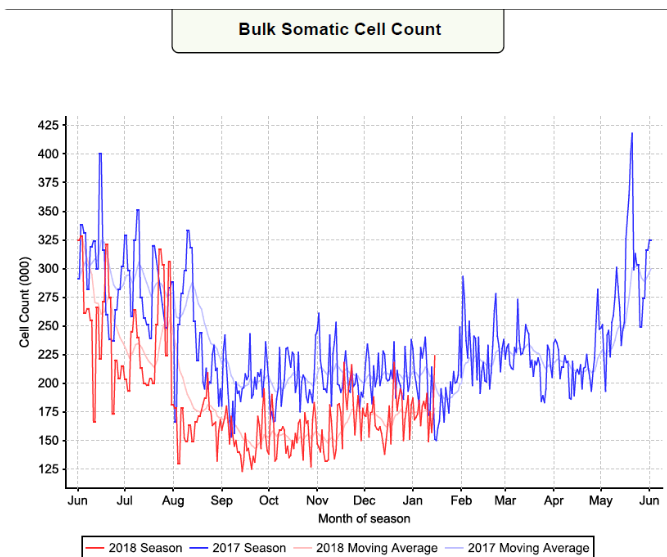
6.2.1 Document 1 BMSCC Curves for Farms 1-4

Figure 6-1 BMSCC Farms 1-4 2018/19 (red), 2017/18 (blue)

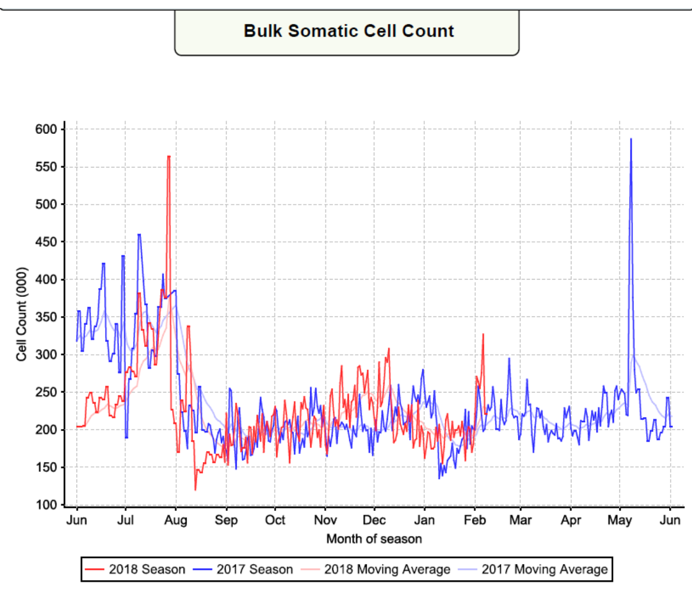
Farm 1



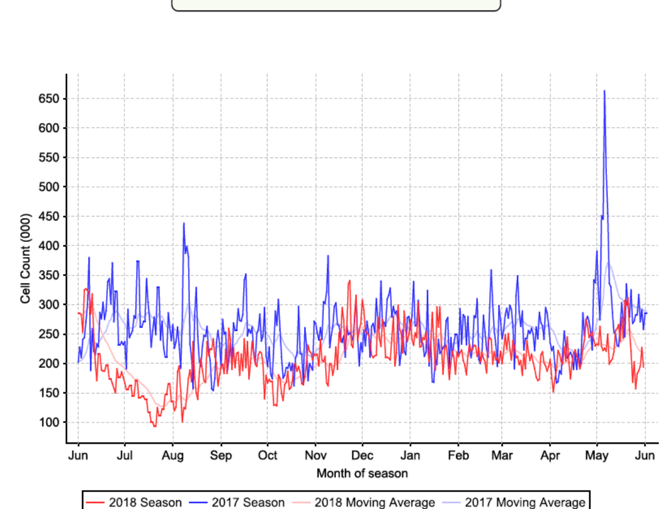
Farm 2



Farm 3



Farm 4



6.2.2 Document 2 Age and Farm Origin

Table 6-1 Age and Farm origin at Surveillance Blood Test 1 Farm 4

	Age	**2**	**3**	**4**	**5**	**6**	**7+**	
Farm of origin	Date	pos	pos	pos	pos	pos	pos	Count
1	19.9.18	0/0	0/10	0/5	0/3	0/0	0/0	18
2	19.9.18	0/0	0/4	0/3	0/3	0/0	0/0	10
3	19.9.18	0/0	0/1	0/4	0/1	0/0	0/0	6
4	19.9.18	0/12	0/11	0/6	0/5	0/0	0/14	48

Table 6-2 Age and Farm origin at Surveillance Blood Test 2 Farm 4

	Age	**2**	**3**	**4**	**5**	**6**	**7+**	
Farm of origin	Date	pos	pos	pos	pos	pos	pos	Count
1	12.10.18	0/0	0/4	0/6	0/1	0/0	0/0	11
2	12.10.18	0/0	0/6	0/0	0/1	0/0	0/0	7
3	12.10.18	0/0	0/0	0/3	0/0	0/1	0/0	4
4	12.10.18	1/10	1/8	1/5	0/4	0/3	0/16	46

6.2.3 Document 3 Apparent and Estimated True Prevalence

Table 6-3 Apparent and Estimated True Prevalence of Farms 1-4 for Programme Surveillance Testing

Farm	Date	Sample size	No pos	App Prev	Lower 95 CI	Upper 95 CI	True Prev	Lower 95 CI	Upper 95 CI
Farm 1	18.9.18	100	33	0.33	0.25	0.43	0.33	0.23	0.45
	10.1.19	133	86	0.65	0.56	0.72	0.71	0.61	0.80
	29.1.19	82	55	0.67	0.56	0.76	0.74	0.61	0.85
Farm 2	18.9.18	100	16	0.16	0.10	0.24	0.13	0.06	0.23
	7.1.19	83	52	0.63	0.52	0.72	0.68	0.56	0.80
	10.1.19	54	40	0.74	0.61	0.84	0.82	0.67	0.94
	11.1.19	93	72	0.77	0.68	0.85	0.86	0.75	0.95
Farm 3	19.9.18	100	3	0.03	0.01	0.08	<0	<0	0.04
	15.10.18	100	14	0.14	0.08	0.22	0.11	0.04	0.20
	24.10.18	100	13	0.13	0.08	0.21	0.09	0.03	0.19
	22.11.18	200	55	0.28	0.22	0.34	0.27	0.20	0.34
	1.2.19	77	53	0.69	0.58	0.78	0.76	0.63	0.87
Farm 4	19.9.18	100	0	0.00	0.00	0.04	<0	<0	<0
	12.10.18	90	3	0.03	0.01	0.09	<0	<0	0.05
	22.2.19	100	1	0.01	0.00	0.05	<0	<0	0.01

6.2.4 Document 4 Historical Information About Cows Milk Sampled at Surveillance

Table 6-4 Historical information about cows milk sampled at surveillance

Assumed management group for cows qPCR milk tested at Surveillance Visit 1 for Farm 1 and 2. (qPCR detect, qPCR not detect)

	Farm 1 (18.9.18)	Milk PCR Det/ND	Farm 2 (18.9.18)	Milk PCR Det/ND
Number milk qPCR detect		16/25		2/24
Colostrum (less than 5 days calved)	9 (incl one unknown cow)	9 ND	10 (incl subclinical IMI 362)	9 ND
Under treatment – or WHP	13	13/13 Det	4	1 Det (957), 3 ND
Assume milkers			4	4/4 ND
Had mastitis, outside milk WHP, assume in herd			5	5/5 ND
Subclinical (PCR Det no treatment)	2 (319 and 852)	2 Det	1 (in colostrum 362)	1 Det
Insufficient Data	1	1 Det (had mastitis earlier?) 917-Rx Aug	1 unknown calving date	1 ND
ELISA positive	12/25 See table below[1]		0/24	

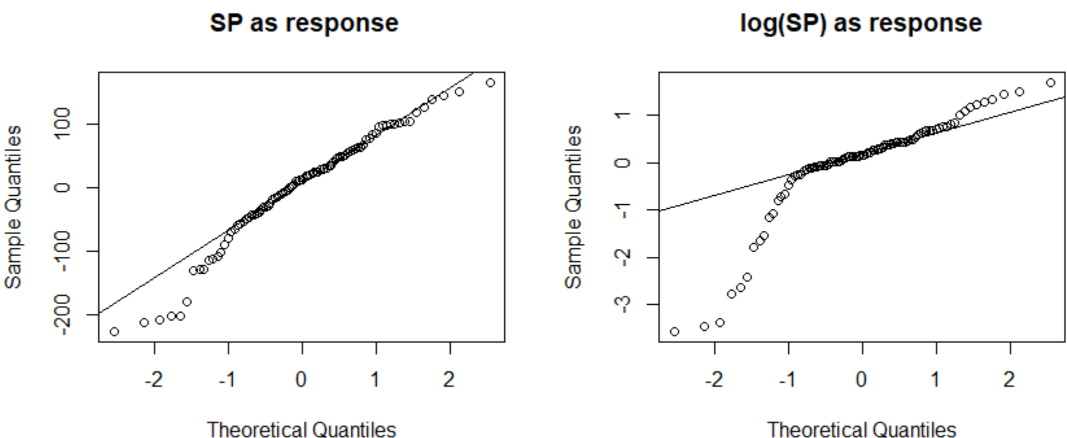
1

	Milk qPCR Det	Milk qPCR ND
ELISA positive	11	1
ELISA negative	5	8

6.2.5 Document 5 Model for SP Values

6.2.5.1 Normality of Plots of Residual for Farms

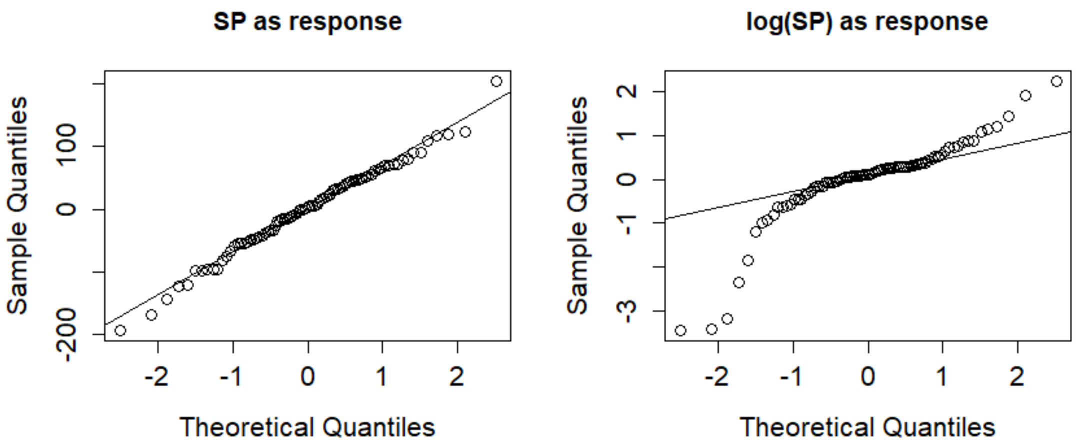
Farm 1



In their modelling of *M. bovis*, Petersen (Petersen et al., 2018) used a log transformation on the response variable (SP%), to improve the normality of model residuals. The residual normality plots above indicate no major problems with normality of residuals with the model using raw SP values as response.

Farm 2

The initial Petersen model included cow ID as a random effect, but this was found to be not significant in the model for this study, having a standard deviation very close to zero. The models used response variables of SP and log(SP).



The additive model was applied using response variable of SP and log(SP) and the normal plots of residuals examined. As with Farm 1 data, the residual normality plots above indicate no problems with normality of residuals with the model using raw SP values as response.

6.2.5.2 Results for the Model with SP% as Response for Farm 1 and Farm 2

Table 6-5 Results of the final general additive model of the association of SP% with estimated time since diagnosis, for Farm 1 and Farm 2

1. Parametric component

	Mean SP% (Estimate)	Std error	t-value	Pr(>|t|)
Farm 1 (Intercept)	213.82	9.23	23.18	<2e-16***
Farm 2 (intercept)	181.44	7.84	23.15	<2e-16***

2. Smooth (non-parametric) component

	edf	Ref. df	F-value	p-value
Farm 1 s(Time1)	3.06	3.80	8.99	7.83e-06***
Farm 2 s(Time2)	3.87	4.78	13.31	<2e-16***

The model output indicates the smooth function of time is a highly significant predictor of SP value, for both Farm 1 and 2. For Farm 1, the estimated degrees of freedom (edf) = 3.06, where a value of 3 indicates a cubic polynomial for the smoothing function. For Farm 2, the edf= 3.84, where a value of 4 indicates a quadratic polynomial of order 4 for the smoothing function.

6.2.6 Document 6 Pathological Findings

Table 6-6 Description of gross morphological lesions, lesion characterisation, special stains & IHC results from 55 M. bovis Clinical Mastitis quarters from 14 dairy cows at slaughter

Cow #	Cow Herd Tag		Cystic Dilation (CD)	Caseous Necrosis (CN)	Fibrosis (F)	Slide	Lesion Characterisation (Histological)	Special Stain Gram Stain	IHC Reactivity
1	906	RF	√	x	√	E528-18/1 E	Suppurative	Neg	Negative
		LF	x	x	√	E528-18/2 E	Suppurative	Neg	Negative
		RH	√	x	√	E528-18/3 E	Granulomatous	Neg	Positive
		LH	√	x	√	E528-18/4 E	Suppurative	Neg	Negative
2	329	RF	√	√	√	E529-18/1 E	Granulomatous	Neg	Positive
		LF	√	√	√	E529-18/2 E	Lymphoplasmacytic	N/A	Negative
		RH		No fixed photo		E529-18/3 E	Granulomatous	Neg	Positive
		LH	√	√	√	E529-18/4 E	Lymphoplasmacytic	N/A	Negative
3	1296	RF	√	√	√	E530-18/1 E	No inflammation	N/A	Negative
		LF	√	√	√	E530-18/2 E	No inflammation	N/A	Negative
		RH	√	√	√	E530-18/3 E	Suppurative	Neg	Negative
		LH	√	√	√	E530-18/4 E	Suppurative	Neg	Negative
4	34	RF	√	√	√	E531-18/1 E	Suppurative	Neg	Positive
		LF	√	√	√	E531-18/2 E	Pyogranulomatous	Neg	Positive
		RH	√	√	√	E531-18/3 E	Suppurative	Neg	Positive
		LH	x	√	√	E531-18/4 E	Granulomatous	Neg	Positive
5	485	RF	√	√	√	E532-18/1 E	Granulomatous	Neg	Positive
		LF	√	√	√	E532-18/2 E	Suppurative	Neg	Suspicious
		RH	√	√	√	E532-18/3 E	Granulomatous	Neg	Positive
		LH	√	√	√	E532-18/4 E	Granulomatous	Neg	Positive
6	1435	RF	√	√	√	E533-18/1 E	No inflammation	N/A	Negative
		LF		Trimmed on slaughter-board		E533-18/2 E			
		RH	√	√	√	E533-18/3 E	No inflammation	N/A	Negative
		LH	√	√	√	E533-18/4 E	Lymphocytic	N/A	Negative
7	426	RF	√	√	√	E534-18/1 E	Pyogranulomatous	Neg	Positive
		LF	√	√	√	E534-18/2 E	Pyogranulomatous	Neg	Positive
		RH	√	√	√	E534-18/3 E	Suppurative	Neg	Positive
		LH	√	√	√	E534-18/4 E	Granulomatous	Neg	Positive
8	557	RF	√	√	√	E536-18/1 E	Granulomatous	Neg	Positive
		LF	√	√	√	E536-18/2 E	Granulomatous	Neg	Positive
		RH	√	√	√	E536-18/3 E	Granulomatous	Neg	Positive
		LH	√	√	√	E536-18/4 E	Granulomatous	Neg	Positive
9	594	RF	√	√	√	E537-18/1 E	Mixed?	N/A	Negative
		LF	√	√	√	E537-18/2 E	Lymphoplasmacytic	N/A	Negative
		RH		No fixed photo		E537-18/3 E	Mixed?	N/A	Negative
		LH	√	√	√	E537-18/4 E	Suppurative	Neg	Negative
10	570	RF		No fixed photo		E538-18/1 E	Suppurative	Neg	Suspicious
		LF	√	√	√	E538-18/2 E	Pyogranulomatous	Neg	Positive
		RH	√	√	√	E538-18/3 E	Pyogranulomatous	Neg	Positive

		LH	√	√	√	E538-18/4 E	Granulomatous	Neg	Positive
11	435	RF	√	√	√	E539-18/1 E	Suppurative	Neg	Negative
		LF	x	x	√	E539-18/2 E	Lymphoplasmacytic	N/A	Negative
		RH	√	√	√	E539-18/3 E	Suppurative	Neg	Negative
		LH	√	x	√	E539-18/4 E	Lymphoplasmacytic	N/A	Negative
12	458	RF	x	x	x	E540-18/1 E	Lymphoplasmacytic	N/A	Negative
		LF	√	x	√	E540-18/2 E	Suppurative	Neg	Negative
		RH	x	x	√	E540-18/3 E	Lymphoplasmacytic	N/A	Negative
		LH	√	√	√	E540-18/4 E	Granulomatous	Neg	Positive
13	50	RF	√	√	√	E541-18/1 E	Pyogranulomatous	Neg	Positive
		LF	√	√	√	E541-18/2 E	Pyogranulomatous	Neg	Positive
		RH	√	x	√	E541-18/3 E	Pyogranulomatous	Neg	Positive
		LH	x	x	√	E541-18/4 E	Suppurative	Neg	Positive
14	655	RF	√	√	√	E542-18/1 E	Granulomatous	Neg	Positive
		LF	√	√	√	E542-18/2 E	Granulomatous	Pos	Positive
		RH	√	√	√	E542-18/3 E	Granulomatous	Neg	Positive
		LH	√	√	√	E542-18/4 E	Granulomatous	Pos	Positive

Chapter 7 References

Adegboye, D., Halbur, P., Cavanaugh, D., Werdin, R., Chase, C., Miskimins, D., & Rosenbusch, R. (1995). Immunohistochemical and pathological study of Mycoplasma bovis-associated lung abscesses in calves. *Journal of Veterinary Diagnostic Investigation, 7*(3), 333-337.

Adegboye, D., Halbur, P., Nutsch, R., Kadlec, R., & Rosenbusch, R. (1996). Mycoplasma bovis-associated pneumonia and arthritis complicated with pyogranulomatous tenosynovitis in calves. *Journal of the American Veterinary Medical Association, 209*(3), 647-649.

Adegboye, D. S., Rasberry, U., Halbur, P. G., Andrews, J. J., & Rosenbusch, R. F. (1995). Monoclonal antibody-based immunohistochemical technique for the detection of Mycoplasma bovis in formalin-fixed, paraffin-embedded calf lung tissues. *Journal of Veterinary Diagnostic Investigation, 7*(2), 261-265.

Aebi, M., Bodmer, M., Frey, J., & Pilo, P. (2012). Herd-specific strains of Mycoplasma bovis in outbreaks of mycoplasmal mastitis and pneumonia. *Veterinary Microbiology, 157*(3-4), 363-368.

Aebi, M., van den Borne, B. H. P., Raemy, A., Steiner, A., Pilo, P., & Bodmer, M. (2015). Mycoplasma bovis infections in Swiss dairy cattle: a clinical investigation. *Acta Veterinaria Scandinavica, 57*, Article 10. https://doi.org/10.1186/s13028-015-0099-x

Al-Abdullah, H., & Fadl, E. A. (2006). Mycoplasma mastitis in dairy cattle herds in Saudi Arabia. *Veterinary Record, 159*(3), 88-89.

Ames, T. R. (1997). Dairy calf pneumonia: the disease and its impact. *Veterinary Clinics: Food Animal Practice, 13*(3), 379-391.

Askaa, G., & Erno, H. (1976). Evaluation of Mycoplasma agalactiae subsp. bovis to species rank Mycoplasma bovis. *International Journal of Systematic Bacteriology, 26*, 323-325.

Ayling, R., Bashiruddin, S., & Nicholas, R. (2004). Mycoplasma species and related organisms isolated from ruminants in Britain between 1990 and 2000. *Veterinary Record, 155*(14), 413-416.

Ayling, R., Rosales, R., Barden, G., & Gosney, F. (2014). Changes in antimicrobial susceptibility of Mycoplasma bovis isolates from Great Britain. *Veterinary Record, 175*(19), 486-486.

Ayling, R. D., Baker, S. E., Nicholas, R. A. J., Peek, M. L., & Simon, A. J. (2000). Comparison of in vitro activity of danofloxacin, florlenicol, oxytetracycline, spectinomycin and tilmicosin against Mycoplasma mycoides subspecies mycoides small colony type. *Veterinary Record, 146*(9), 243-246. https://doi.org/10.1136/vr.146.9.243

Bashiruddin, J. B., Frey, J., Königsson, M. H., Johansson, K.-E., Hotzel, H., Diller, R., De Santis, P., Botelho, A., Ayling, R. D., & Nicholas, R. A. (2005). Evaluation of PCR systems for the identification and differentiation of Mycoplasma agalactiae and Mycoplasma bovis: a collaborative trial. *The Veterinary Journal, 169*(2), 268-275.

Bayoumi, F., Farver, T. B., Bushnell, B., & Oliveria, M. (1988). Enzootic mycoplasmal mastitis in a large dairy during an eight-year period. *Journal of the American Veterinary Medical Association, 192*(7), 905-909.

Beckman, J. S., & Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *American Journal of Physiology-cell physiology, 271*(5), C1424-C1437.

Behrens, A., Heller, M., Kirchhoff, H., Yogev, D., & Rosengarten, R. (1994). A family of phase- and size-variant membrane surface lipoprotein antigens (Vsps) of Mycoplasma bovis. *Infection and immunity, 62*(11), 5075-5084.

Behrens, A., Poumarat, F., Le Grand, D., Heller, M., & Rosengarten, R. (1996). A newly identified immunodominant membrane protein (pMB67) involved in Mycoplasma bovis surface antigenic variation. *Microbiology, 142*(9), 2463-2470.

Benites, N., Guerra, J., Melville, P., & Da Costa, E. (2002). Aetiology and histopathology of bovine mastitis of espontaneous occurrence. *Journal of Veterinary Medicine, Series B, 49*(8), 366-370.

Bennett, R., & Jasper, D. (1978). Systemic and local immune responses associated with bovine mammary infections due to Mycoplasma bovis: resistance and susceptibility in previously infected cows. *Am J Vet Research, 39*, 417-423.

Bennett, R., & Jasper, D. (1980). Bovine mycoplasmal mastitis from intramammary inoculations of small numbers of Mycoplasma bovis: local and systemic antibody response. *American Journal of Veterinary Research, 41*(6), 889-892.

Bennett, R. H., & Jasper, D. E. (1977a). Bovine mycoplasma mastitis from intramammary inoculation of small numbers of Mycoplasma bovis. I. Microbiology and pathology. *Veterinary Microbiology, 2*(4), 341-355. https://doi.org/https://doi.org/10.1016/0378-1135(77)90044-X

Bennett, R. H., & Jasper, D. E. (1977b). Nasal prevalence of Mycoplasma bovis and IHA titers in young dairy animals. *The Cornell Veterinarian, 67*(3), 361-373.

Bianchi, R. M., Schwertz, C. I., de Cecco, B. S., Panziera, W., De Lorenzo, C., Heck, L. C., Snel, G. G., Lopes, B. C., da Silva, F. S., & Pavarini, S. P. (2019). Pathological and microbiological characterization of mastitis in dairy cows. *Tropical animal health and production, 51*(7), 2057-2066.

Bicknell, S., Gunning, R., Jackson, G., Boughton, E., & Wilson, C. (1983). Eradication of Mycoplasma bovis infection from a dairy herd in Great Britain. *The Veterinary record, 112*(13), 294-297.

Bicknell, S., Jackson, G., Boughton, E., & Wilson, C. (1978). Mycoplasma bovis mastitis in the dry cow. *The Veterinary record, 102*(17), 389. https://doi.org/doi: 10.1136/vr.102.17.389-a

Biddle, M. K., Fox, L. K., Evans, M. A., & Gay, C. C. (2005). Pulsed-field gel electrophoresis patterns of Mycoplasma isolates from various body sites in dairy cattle with Mycoplasma mastitis. *Journal of the American Veterinary Medical Association, 227*(3), 455-459.

Biddle, M. K., Fox, L. K., & Hancock, D. D. (2003). Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. *Journal of the American Veterinary Medical Association, 223*(8), 1163-1166.

Bielanski, A., Devenish, J., & Phipps-Todd, B. (2000). Effect of Mycoplasma bovis and Mycoplasma bovigenitalium in semen on fertilization and association with in vitro produced morula and blastocyst stage embryos. *Theriogenology, 53*(6), 1213-1223.

Biosecurity New Zealand. (2018a). *Phased Eradication of Mycoplasma Bovis*. Ministry for Primary Industries. https://www.mpi.govt.nz/dmsdocument/29303-phased-eradication-of-mycoplasma-bovis-fact-sheet

Biosecurity New Zealand. (2018b). *Science Group to help Mycoplasma bovis eradication efforts*. https://www.mpi.govt.nz/news-and-resources/media-releases/science-group-to-help-mycoplasma-bovis-eradication-efforts/

Biosecurity New Zealand. (2020). *Active Confirmed Property. Information pack for farmers*. https://www.mbovis.govt.nz/assets/Uploads/Active-Confirmed-Property-Information-Pack-version-January-2020.pdf

Boonyayatra, S., Fox, L., Besser, T., Sawant, A., Gay, J., & Raviv, Z. (2012). A PCR assay and PCR-restriction fragment length polymorphism combination identifying the 3 primary Mycoplasma species causing mastitis. *Journal of Dairy Science, 95*(1), 196-205.

Boonyayatra, S., Fox, L., Besser, T. E., Sawant, A., & Gay, J. (2010). Effects of storage methods on the recovery of Mycoplasma species from milk samples. *Veterinary Microbiology, 144*(1-2), 210-213.

Boothby, J., Jasper, D., & Thomas, C. (1986). Experimental intramammary inoculation with Mycoplasma bovis in vaccinated and unvaccinated cows: effect on the mycoplasmal infection and cellular inflammatory response. *The Cornell Veterinarian, 76*(2), 188-197.

Bradley, A., Leach, K., Breen, J., Green, L., & Green, M. (2007). Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Veterinary Record, 160*(8), 253-258.

Brookbanks E, Carter M. E., & Holland J. T. S. (1969). Mycoplasma mastitis. *New Zealand Veterinary Journal, 17*(9), 179-180. https://doi.org/10.1080/00480169.1969.33818

Brown, M., Dechant, D., Donovan, G., Hutchinson, J., & Brown, D. (1998). Association of Mycoplasma bovis with otitis media in dairy calves. *IOM Lett, 12*, 104-105.

Brown, M., Shearer, J., & Elvinger, F. (1990). Mycoplasmal mastitis in a dairy herd. *Journal of the American Veterinary Medical Association, 196*(7), 1097-1101.

Brys, A., & Pfützner, H. (1989). Testing of a hyperimmune serum in calves infected intranasally with Mycoplasma bovis. *Archiv fur experimentelle Veterinarmedizin, 43*(5), 677-683.

Brys, A., Pfutzner, H., & Bocklisch, H. (1992). Mycoplasma-bovis-freie Aufzucht von Rindern. *Berl Munch Tierarztl Wochenschr, 105*, 230-232.

Buchenau, I., Poumarat, F., Le Grand, D., Linkner, H., Rosengarten, R., & Hewicker-Trautwein, M. (2010). Expression of Mycoplasma bovis variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of Mycoplasma bovis type strain PG45. *Research in veterinary science, 89*(2), 223-229.

Buckle, K., Castillo-Alcala, F., Ha, H., Begg, D., McDonald, W., & Bingham, P. (2020). First report of the use of mucosal swabs of the palatine tonsillar crypt for detection of Mycoplasma bovis in naturally infected calves. *New Zealand Veterinary Journal*(just-accepted), 1-7.

Bürki, S., Frey, J., & Pilo, P. (2015). Virulence, persistence and dissemination of Mycoplasma bovis. *Veterinary Microbiology, 179*(1-2), 15-22.

Bürki, S., Spergser, J., Bodmer, M., & Pilo, P. (2016). A dominant lineage of Mycoplasma bovis is associated with an increased number of severe mastitis cases in cattle. *Veterinary Microbiology, 196*, 63-66.

Burnens, A., Bonnemain, P., Bruderer, U., Schalch, L., Audige, L., Le Grand, D., Poumarat, F., & Nicolet, J. (1999). Study on the seroprevalence of infections by Mycoplasma bovis in Swiss cattle including epidemiological analysis of risk factors in a local population (Jura region). *Schweizer Archiv Fur Tierheilkunde, 141*(10), 455-460.

Bush, T. J. V., & Rosenbusch, R. F. (2003). Characterization of the immune response to Mycoplasma bovis lung infection. *Veterinary Immunology and Immunopathology, 94*(1-2), 23-33.

Bushnell, R. B. (1984). Mycoplasma mastitis. *The Veterinary clinics of North America. Large animal practice, 6*(2), 301-312.

Byrne, W., Ball, H., McCormack, R., & Brice, N. (1998). Elimination of Mycoplasma bovis mastitis from an Irish dairy herd. *The Veterinary record, 142*(19), 516-517.

Byrne, W., McCormack, R., Ball, H., Brice, N., Baker, S., Ayling, R., & Nicholas, R. (2000). Application of an indirect ELISA to milk samples to identify cows with Mycoplasma bovis mastitis. *Veterinary Record, 146*(13), 368-369.

Cai, H. Y., Bell-Rogers, P., Parker, L., & Prescott, J. F. (2005). Development of a real-time PCR for detection of Mycoplasma bovis in bovine milk and lung samples. *Journal of Veterinary Diagnostic Investigation, 17*(6), 537-545.

Calcutt, M., Lysnyansky, I., Sachse, K., Fox, L., Nicholas, R., & Ayling, R. (2018). Gap analysis of Mycoplasma bovis disease, diagnosis and control: an aid to identify future development requirements. *Transboundary and emerging diseases, 65*, 91-109.

Chalker, V., & Spiller, B. (2019). Abstracts. European Mycoplasma Conference, London.

Cho, H., Ruhnke, H., & Langford, E. (1976). The indirect hemagglutination test for the detection of antibodies in cattle naturally infected mycoplasmas. *Canadian Journal of Comparative Medicine, 40*(1), 20-29.

Clothier, K. A., Jordan, D. M., Thompson, C. J., Kinyon, J. M., Frana, T. S., & Strait, E. L. (2010). Mycoplasma bovis real-time polymerase chain reaction assay validation and diagnostic performance. *Journal of Veterinary Diagnostic Investigation, 22*(6), 956-960.

Corbellini, L., Driemeier, D., Cruz, C., Dias, M., & Ferreiro, L. (2001). Bovine mastitis due to Prototheca zopfii: clinical, epidemiological and pathological aspects in a Brazilian dairy herd. *Tropical animal health and production, 33*(6), 463-470.

DairyNZ. (2017a). Calving Pattern. In M. Blackwell, K. Roberts, R. Ellison, & J. Penry (Eds.), *The InCalf book for New Zealand dairy farmers* (2nd ed., pp. 61-67). DairyNZ Ltd.

DairyNZ. (2017b). Pasture-based production systems. In *Facts and Figures. A quick reference guide for New Zealand dairy farmers* (2nd ed., pp. 23-43). DairyNZ Ltd.

Dijkshoorn, L., Ursing, B., & Ursing, J. (2000). Strain, clone and species: comments on three basic concepts of bacteriology. *Journal of Medical Microbiology, 49*(5), 397-401.

Dohoo, I., Martin, W., Styhn, H. (2014). *Veterinary Epidemiologic Research* (2nd ed.). VER Inc.

El-Sayed, A., Awad, W., Abdou, N.-E., & Vázquez, H. C. (2017). Molecular biological tools applied for identification of mastitis causing pathogens. *International journal of veterinary science and medicine, 5*(2), 89-97.

Enger, B. D., Fox, L. K., Gay, J. M., & Johnson, K. A. (2015). Reduction of teat skin mastitis pathogen loads: Differences between strains, dips, and contact times. *Journal of Dairy Science, 98*(2), 1354-1361. https://doi.org/10.3168/jds.2014-8622

Enøe, C., Georgiadis, M. P., & Johnson, W. O. (2000). Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease state is unknown. *Preventive veterinary medicine, 45*(1-2), 61-81.

Feenstra, A., Madsen, E. B., Friis, N., Meyling, A., & Ahrens, P. (1991). A field study of Mycoplasma bovis infection in cattle. *Journal of Veterinary Medicine, Series B, 38*(1-10), 195-202.

Filioussis, G., Christodoulopoulos, G., Thatcher, A., Petridou, V., & Bourtzi-Chatzopoulou, E. (2007). Isolation of Mycoplasma bovis from bovine clinical mastitis cases in Northern Greece. *The Veterinary Journal, 173*(1), 215-218.

Fink-Gremmels, J. (2008). The role of mycotoxins in the health and performance of dairy cows. *The Veterinary Journal, 176*(1), 84-92.

Fligger, J., Waldvogel, A., Pfister, H., & Jungi, T. (1999). Expression of inducible nitric oxide synthase in spontaneous bovine bronchopneumonia. *Veterinary pathology, 36*(5), 397-405.

Foster, A. P., Naylor, R. D., Howie, N. M., Nicholas, R. A., & Ayling, R. D. (2009). Mycoplasma bovis and otitis in dairy calves in the United Kingdom. *Vet J, 179*(3), 455-457. https://doi.org/10.1016/j.tvjl.2007.10.020

Fox, L., Hancock, D., Mickelson, A., Britten, A., & Kaaden, O. R. (2003). Bulk tank milk analysis: factors associated with appearance of Mycoplasma sp. in milk. *Journal of Veterinary Medicine, Series B, 50*(5), 235-240.

Fox, L. K. (2012). Mycoplasma Mastitis Causes, Transmission, and Control. *Veterinary Clinics of North America-Food Animal Practice, 28*(2), 225-237. https://doi.org/10.1016/j.cvfa.2012.03.007

Fox, L. K., Kirk, J. H., & Britten, A. (2005). Mycoplasma mastitis: A review of transmission and control. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health, 52*(4), 153-160. https://doi.org/10.1111/j.1439-0450.2005.00845.x

Fox, L. K., Muller, F. J., Wedam, M. L., Schneider, C. S., & Biddle, M. K. (2008). Clinical Mycoplasma bovis mastitis in prepubertal heifers on 2 dairy herds. *The Canadian Veterinary Journal, 49*(11), 1110-1112.

Freundt, E., & Edward, D. (1971). International Committee on Nomenclature of Bacteria Subcommittee on the Taxonomy of Mycoplasmatales: Minutes of the Meeting, 10 August 1970. *International Journal of Systematic and Evolutionary Microbiology, 21*(1), 151-153.

Frost, A., Wanasinghe, D., & Woolcock, J. (1977). Some factors affecting selective adherence of microorganisms in the bovine mammary gland. *Infection and immunity, 15*(1), 245-253.

Gagea, M. I., Bateman, K. G., Shanahan, R. A., Van Dreumel, T., McEwen, B. J., Carman, S., Archambault, M., & Caswell, J. L. (2006). Naturally occurring Mycoplasma bovis—associated pneumonia and polyarthritis in feedlot beef calves. *Journal of Veterinary Diagnostic Investigation, 18*(1), 29-40.

Gans, L. H. (2020). *Morphologic and immunohistochemical characterisation of Mycoplasma bovis mastitis in dairy cows in New Zealand* [Unpublished Masters thesis]. Massey University.

Gautier-Bouchardon, A. V., Ferre, S., Le Grand, D., Paoli, A., Gay, E., & Poumarat, F. (2014). Overall decrease in the susceptibility of Mycoplasma bovis to antimicrobials over the past 30 years in France. *PLoS One, 9*(2).

Ghadersohi, A., Hirst, R., Forbes-Faulkener, J., & Coelen, R. (1999). Preliminary studies on the prevalence of Mycoplasma bovis mastitis in dairy cattle in Australia. *Veterinary Microbiology, 65*(3), 185-194.

Ghazaei, C. (2006). Mycoplasmal mastitis in dairy cows in the Moghan region of Ardabil State, Iran. *Journal of the South African Veterinary Association, 77*(4), 222-223.

Gille, L., Callens, J., Supre, K., Boyen, F., Haesebrouck, F., Van Driessche, L., van Leenen, K., Deprez, P., & Pardon, B. (2018). Use of a breeding bull and absence of a calving pen as risk factors for the presence of Mycoplasma bovis in dairy herds. *J Dairy Sci, 101*(9), 8284-8290. https://doi.org/10.3168/jds.2018-14940

Gillespie, B., & Oliver, S. (2005). Simultaneous detection of mastitis pathogens, Staphylococcus aureus, Streptococcus uberis, and Streptococcus agalactiae by multiplex real-time polymerase chain reaction. *Journal of Dairy Science, 88*(10), 3510-3518.

Gioia, G., Werner, B., Nydam, D., & Moroni, P. (2016). Validation of a mycoplasma molecular diagnostic test and distribution of mycoplasma species in bovine milk among New York State dairy farms. *Journal of Dairy Science, 99*(6), 4668-4677.

Godden, S. M., Jansen, J. T., Leslie, K. E., Smart, N. L., & Kelton, D. F. (2002). The effect of sampling time and sample handling on the detection of Staphylococcus aureus in milk from quarters with subclinical mastitis. *The Canadian Veterinary Journal, 43*(1), 38.

Godinho, K., Sarasola, P., Renoult, E., Tilt, N., Keane, S., Windsor, G., Rowan, T., & Sunderland, S. (2007). Use of deep nasopharyngeal swabs as a predictive diagnostic method for natural respiratory infections in calves. *Veterinary Record, 160*, 22-25.

Goff, J. (2006). Major advances in our understanding of nutritional influences on bovine health. *Journal of Dairy Science, 89*(4), 1292-1301.

Gomes, F., Saavedra, M. J., & Henriques, M. (2016). Bovine mastitis disease/pathogenicity: evidence of the potential role of microbial biofilms. *Pathogens and disease, 74*(3), 1-7.

González, R. (1999). Mamitis micoplástica en establecimientos lecheros: un enfoque holístico a su diagnóstico y control. *Bovis*(86), 77-91.

González, R., Jayarao, B., Oliver, S., & Sears, P. (1993). Pneumonia, arthritis and mastitis in dairy cows due to Mycoplasma bovis. Annual meeting (USA),

Gonzalez, R., Merrill, R., & Sears, P. (1992). Shedding of Mycoplasma bovis from the udder of naturally infected cows and its importance for the diagnosis of bovine intramammary infections. *J Dairy Sci, 75*(Suppl 1), 259.

González, R. N., & Wilson, D. J. (2003). Mycoplasmal mastitis in dairy herds. *Veterinary Clinics: Food Animal Practice, 19*(1), 199-221.

González, Y. R. C., Bascuñana, C. R., Bölske, G., Mattsson, J. G., Molina, C. F., & Johansson, K.-E. (1995). In vitro amplification of the 16S rRNA genes from Mycoplasma bovis and Mycoplasma agalactiae by PCR. *Veterinary Microbiology, 47*(1-2), 183-190.

Government, N. Z. (1993). *New Zealand Legislation Biosecurity Act 1993*. New Zealand Parliamentary Counsel office. http://legislation.govt.nz/act/public/1993/0095/DLM314623.html

Graber, H. U., Casey, M., Naskova, J., Steiner, A., & Schaeren, W. (2007). Development of a highly sensitive and specific assay to detect Staphylococcus aureus in bovine mastitic milk. *Journal of Dairy Science, 90*(10), 4661-4669.

Green, R., Newmark, P. A., Mussos, A. M., & Mollin, D. L. (1974). The use of chicken serum for measurement of serum vitamin B12 concentration by radioisotope dilution: description of method and comparison with microbiological assay results. *British Journal of Haematology, 27*(3), 507-526.

Greiner, M., & Gardner, I. (2000). Application of diagnostic tests in veterinary epidemiologic studies. *Preventive veterinary medicine, 45*(1-2), 43-59.

Gwet, K. L. (2008). Computing inter-rater reliability and its variance in the presence of high agreement. *Br J Math Stat Psychol, 61*(Pt 1), 29-48. https://doi.org/10.1348/000711006X126600

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., & Autio, T. (2018). Semen as a source of Mycoplasma bovis mastitis in dairy herds. *Veterinary Microbiology, 216*, 60-66.

Hale, H., Helmboldt, C., Plastridge, W., & Stula, E. (1962). Bovine mastitis caused by a Mycoplasma species. *The Cornell Veterinarian, 52*, 582-591.

Hames, C., Halbedel, S., Hoppert, M., Frey, J., & Stülke, J. (2009). Glycerol metabolism is important for cytotoxicity of Mycoplasma pneumoniae. *Journal of bacteriology, 191*(3), 747-753.

Hata, E., Suzuki, K.-i., Hanyu, H., Itoh, M., Higuchi, H., & Kobayashi, H. (2014). Molecular epidemiology of cases of Mycoplasma californicum infection in Japan. *Appl. Environ. Microbiol., 80*(24), 7717-7724.

Hay, M. (2018). Mycoplasma bovis infection on a South Canterbury dairy farm. *Conference Proceedings of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*, 69-72.

Hazelton, M., Morton, J., Parker, A., Sheehy, P., Bosward, K., Malmo, J., & House, J. (2020). Whole dairy herd sampling to detect subclinical intramammary Mycoplasma bovis infection after clinical mastitis outbreaks. *Veterinary Microbiology*, 108662.

Hazelton, M., Sheehy, P., Bosward, K., Parker, A., Morton, J., Dwyer, C., Niven, P., & House, J. (2018). Shedding of Mycoplasma bovis and antibody responses in cows recently diagnosed with clinical infection. *Journal of Dairy Science, 101*(1), 584-589.

Hazlett, M., Little, P., Maxie, M., & Barnum, D. (1984). Fatal mastitis of dairy cows: a retrospective study. *Canadian Journal of Comparative Medicine, 48*(2), 125.

Helmboldt, C. F., Jungherr, E. L., & Plastridge, W. N. (1953). Histopathology of Bovine Mastitis, The. *Storrs Agricultural Experiment Station. 45*. https://opencommons.uconn.edu/saes/45

Hermann R. (1992). Genome structure and organisation. In J. Maniloff (Ed.), *Mycoplasmas: molecular biology and pathogenesis.* (pp. 157-168). American Society of Microbiology.
Hermeyer, K., Jacobsen, B., Spergser, J., Rosengarten, R., & Hewicker-Trautwein, M. (2011). Detection of Mycoplasma bovis by in-situ hybridization and expression of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase in the lungs of experimentally-infected calves. *Journal of comparative pathology, 145*(2-3), 240-250.
Hermeyer, K., Peters, M., Brügmann, M., Jacobsen, B., & Hewicker-Trautwein, M. (2012). Demonstration of Mycoplasma bovis by immunohistochemistry and in situ hybridization in an aborted bovine fetus and neonatal calf. *Journal of Veterinary Diagnostic Investigation, 24*(2), 364-369.
Hertl, J., Schukken, Y., Bar, D., Bennett, G., González, R., Rauch, B., Welcome, F., Tauer, L., & Gröhn, Y. (2011). The effect of recurrent episodes of clinical mastitis caused by gram-positive and gram-negative bacteria and other organisms on mortality and culling in Holstein dairy cows. *Journal of Dairy Science, 94*(10), 4863-4877.
Heuvelink, A., Reugebrink, C., & Mars, J. (2016). Antimicrobial susceptibility of Mycoplasma bovis isolates from veal calves and dairy cattle in the Netherlands. *Veterinary Microbiology, 189*, 1-7.
Higuchi, H., Gondaira, S., Iwano, H., Hirose, K., Nakajima, K., Kawai, K., Hagiwara, K., Tamura, Y., & Nagahata, H. (2013). Mycoplasma species isolated from intramammary infection of Japanese dairy cows. *Veterinary Record, 172*(21), 557-557.
Hirose, K., Kawasaki, Y., Kotani, K., Tanaka, A., Abiko, K., & Ogawa, H. (2001). Detection of mycoplasma in mastitic milk by PCR analysis and culture method. *Journal of Veterinary Medical Science, 63*(6), 691-693.
Hodges R, MacPherson M., Leach R. H., & Moller S. (1983). Isolation of Mycoplasma dispar from mastitis in dry cows. *New Zealand Veterinary Journal, 31*(4), 60-61. https://doi.org/10.1080/00480169.1983.34967
Houlihan, M., Veenstra, B., Christian, M., Nicholas, R., & Ayling, R. (2007). Mastitis and arthritis in two dairy herds caused by Mycoplasma bovis. *The Veterinary record, 160*(4), 126-127.
Hui, S. L., & Walter, S. D. (1980). Estimating the error rates of diagnostic tests. *Biometrics*, 167-171.
Hum, S., Kessell, A., Djordjevic, S., Rheinberger, R., Hornitzky, M., Forbes, W., & Gonsalves, J. (2000). Mastitis, polyarthritis and abortion caused by Mycoplasma species bovine group 7 in dairy cattle. *Australian Veterinary Journal, 78*(11), 744-750.
Hussain, R., Javed, M. T., Khan, A., Mahmood, F., & Kausar, R. (2012). Mastitis and associated histo-pathological consequences in the context of udder morphology. *International Journal of Agriculture and Biology, 14*(6).
Huzzey, J., Nydam, D., Ospina, P., & Overton, T. (2012). *Predicting transition cow health and performance-use of blood and fecal biomarkers for herd-level evaluation and diagnostics* Northeast Dairy Producers Association (NEDPA), Syracuse, New York, USA. https://hdl.handle.net/1813/36568
ID.vet. (2018). *Internal Validation Report ID Screen Mycoplasma bovis Indirect* https://www.id-vet.com/produit/id-screen-mycoplasma-bovis-indirect/
Imada, Y., Uchida, I., & Hashimoto, K. (1987). Rapid identification of mycoplasmas by indirect immunoperoxidase test using small square filter paper. *Journal of clinical microbiology, 25*(1), 17-21.
Jain, N., Jasper, D., & Dellinger, J. (1967). Cultural characters and serological relationships of some mycoplasmas isolated from bovine sources. *Microbiology, 49*(3), 401-410.

Jain, N., Jasper, D., & Dellinger, J. (1969). Experimental bovine mastitis due to mycoplasma. *The Cornell Veterinarian, 59*(1), 10-28.

Jasper, D., & Al Aubaidi, J. (1974). Epidemiologic observations on mycoplasma mastitis. *The Cornell Veterinarian, 64*(4), 407-415.

Jasper, D. E. (1981a). Bovine mycoplasmal mastitis. *Advances in veterinary science and comparative medicine, 25*, 121-159.

Jasper, D. E. (1981b). Bovine mycoplasmal mastitis. *Advances in veterinary science and comparative medicine, 25*, 121-157.

Johnstone, A., & King, C. (2003). Bovine and ovine Mycoplasmal arthritis and synovitis. *New Zealand Veterinary Journal, 51*(1), 45-46.

Jones, T., & Hunt, R. (1983). *Veterinary Pathology*. Lea and Febiger, Philadelphia. PA. USA.

Jungi, T., Krampe, M., Sileghem, M., Griot, C., & Nicolet, J. (1996). Differential and strain-specific triggering of bovine alveolar macrophage effector functions by mycoplasmas. *Microbial pathogenesis, 21*(6), 487-498.

Justice-Allen, A., Trujillo, J., Corbett, R., Harding, R., Goodell, G., & Wilson, D. (2010). Survival and replication of Mycoplasma species in recycled bedding sand and association with mastitis on dairy farms in Utah. *Journal of Dairy Science, 93*(1), 192-202.

Kauf, A., Rosenbusch, R., Paape, M., & Bannerman, D. D. (2007). Innate immune response to intramammary Mycoplasma bovis infection. *Journal of Dairy Science, 90*(7), 3336-3348.

Keane, O. M., Budd, K. E., Flynn, J., & McCoy, F. (2013). Increased detection of mastitis pathogens by real-time PCR compared to bacterial culture. *Veterinary Record, 173*(11), 268-268.

Khan, L. A., Loria, G. R., Ramirez, A. S., Nicholas, R. A., Miles, R. J., & Fielder, M. D. (2005). Biochemical characterisation of some non fermenting, non arginine hydrolysing mycoplasmas of ruminants. *Veterinary Microbiology, 109*(1-2), 129-134.

Kleinschmidt, S., Spergser, J., Rosengarten, R., & Hewicker-Trautwein, M. (2013). Long-term survival of Mycoplasma bovis in necrotic lesions and in phagocytic cells as demonstrated by transmission and immunogold electron microscopy in lung tissue from experimentally infected calves. *Veterinary Microbiology, 162*(2-4), 949-953.

Kumar, A., Rahal, A., Chakraborty, S., Verma, A. K., & Dhama, K. (2014). Mycoplasma agalactiae, an etiological agent of contagious agalactia in small ruminants: a review. *Veterinary medicine international, 2014*.

Kusiluka, L. J., Kokotovic, B., Ojeniyi, B., Friis, N. F., & Ahrens, P. (2000). Genetic variations among Mycoplasma bovis strains isolated from Danish cattle. *FEMS microbiology letters, 192*(1), 113-118.

Kusiluka, L. J., Ojeniyi, B., & Friis, N. (2000). Increasing Prevalence of Mycoplasma bovis in Danish Cattle. *Acta vet. scand, 41*, 139-146.

LaFaunce, N., & McEntee, K. (1982). Experimental Mycoplasma bovis seminal vesiculitis in the bull. *The Cornell Veterinarian, 72*(2), 150-167.

Lamm, C. G., Munson, L., Thurmond, M. C., Barr, B. C., & George, L. W. (2004). Mycoplasma otitis in California calves. *Journal of Veterinary Diagnostic Investigation, 16*(5), 397-402.

Lanzas, C., Brien, S., Ivanek, R., Lo, Y., Chapagain, P., Ray, K., Ayscue, P., Warnick, L., & Gröhn, Y. (2008). The effect of heterogeneous infectious period and contagiousness on the dynamics of Salmonella transmission in dairy cattle. *Epidemiology & Infection, 136*(11), 1496-1510.

Le Grand, D., Bézille, P., Calavas, D., Poumarat, F., Brank, M., Citti, C., & Rosengarten, R. (2002). Serological prevalence of Mycoplasma bovis infection in suckling beef cattle in France. *Veterinary Record, 150*(9), 268-273.

Levisohn, S., Garazi, S., Gerchman, I., & Brenner, J. (2004). Diagnosis of a mixed mycoplasma infection associated with a severe outbreak of bovine pinkeye in young calves. *Journal of Veterinary Diagnostic Investigation, 16*(6), 579-581.

Lopez-Benavides, M., Dohoo, I., Scholl, D., Middleton, J., & Perez, R. (2018). Interpreting Bacteriological Culture Results to Diagnose Bovine Intramammary Infections. *NMC Research Committee Report*.

Lysnyansky, I., Freed, M., Rosales, R. S., Mikula, I., Khateb, N., Gerchman, I., van Straten, M., & Levisohn, S. (2016). An overview of Mycoplasma bovis mastitis in Israel (2004–2014). *The Veterinary Journal, 207*, 180-183.

Lysnyansky, I., Rosengarten, R., & Yogev, D. (1996). Phenotypic switching of variable surface lipoproteins in Mycoplasma bovis involves high-frequency chromosomal rearrangements. *Journal of bacteriology, 178*(18), 5395-5401.

Lysnyansky, I., Sachse, K., Rosenbusch, R., Levisohn, S., & Yogev, D. (1999). The vsp locus of Mycoplasma bovis: gene organization and structural features. *Journal of bacteriology, 181*(18), 5734-5741.

Mackereth, G., & Marquetoux, N. (2019). *Evaluation of the IDVET serum ELISA on bovine serum in the New Zealand Mycoplasma bovis eradication programme.* [Unpublished]. Ministry for Primary Industries.

Mah, T.-F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in microbiology, 9*(1), 34-39.

Maunsell, F., Brown, M. B., Powe, J., Ivey, J., Woolard, M., Love, W., & Simecka, J. W. (2012). Oral inoculation of young dairy calves with Mycoplasma bovis results in colonization of tonsils, development of otitis media and local immunity. *PLoS One, 7*(9).

Maunsell, F. P., & Donovan, G. A. (2009). Mycoplasma bovis infections in young calves. *Veterinary Clinics of North America: Food Animal Practice, 25*(1), 139-177.

Maunsell, F. P., Woolums, A. R., Francoz, D., Rosenbusch, R. F., Step, D. L., Wilson, D. J., & Janzen, E. D. (2011). Mycoplasma bovis Infections in Cattle. *Journal of Veterinary Internal Medicine, 25*(4), 772-783. https://doi.org/10.1111/j.1939-1676.2011.0750.x

McAuliffe, L., Ellis, R. J., Lawes, J. R., Ayling, R. D., & Nicholas, R. A. (2005). 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating Mycoplasma species. *Journal of Medical Microbiology, 54*(8), 731-739.

McAuliffe, L., Ellis, R. J., Miles, K., Ayling, R. D., & Nicholas, R. A. (2006). Biofilm formation by mycoplasma species and its role in environmental persistence and survival. *Microbiology, 152*(4), 913-922.

McAuliffe, L., Kokotovic, B., Ayling, R. D., & Nicholas, R. A. (2004). Molecular epidemiological analysis of Mycoplasma bovis isolates from the United Kingdom shows two genetically distinct clusters. *Journal of clinical microbiology, 42*(10), 4556-4565.

McCluskey, B. J., Lombard, J. E., & Hirst, H. (2003). Mycoplasma in bulk tank milk on US dairy operations. *Proceedings of the 10th International Symposium for Veterinary Epidemiology and Economics: Vina del Mar, Chile.* http://hdl.handle.net/10113/44109.

McDougall, S., Arthur, D., Bryan, M., Vermunt, J., & Weir, A. (2007). Clinical and bacteriological response to treatment of clinical mastitis with one of three intramammary antibiotics. *New Zealand Veterinary Journal, 55*(4), 161-170.

McDougall, S., Bryan, M., & Tiddy, R. (2009). Effect of treatment with the nonsteroidal antiinflammatory meloxicam on milk production, somatic cell count, probability of re-treatment, and culling of dairy cows with mild clinical mastitis. *Journal of Dairy Science, 92*(9), 4421-4431.

McFadden, A., Ha, H., Donald, J., Bueno, I., van Andel, M., Thompson, J., Tisdall, D., & Pulford, D. (2016). Investigation of bovine haemoplasmas and their association with anaemia in New Zealand cattle. *New Zealand Veterinary Journal, 64*(1), 65-68.

Miles, K., McAuliffe, L., Persson, A., Ayling, R. D., & Nicholas, R. A. (2005). Insertion sequence profiling of UK Mycoplasma bovis field isolates. *Veterinary Microbiology, 107*(3-4), 301-306.

Millar, K., Albyt, A., & Bond, G. (1984). Measurement of vitamin B12 in the livers and sera of sheep and cattle and an investigation of factors influencing serum vitamin B12 levels in sheep. *New Zealand Veterinary Journal, 32*(5), 65-70.

Ministry for Primary Industries. (2015). *Operational Code for Presentation of Post Mortem Examination. Red Meat Code of Practice Chapter 6.*

Ministry for Primary Industries. (2018). *Mycoplasma bovis Science Plan*. https://www.mpi.govt.nz/dmsdocument/32713-mycoplasma-bovis-science-plan

Ministry for Primary Industries. (2019a). *Mycoplasma bovis 2019 National Plan*. https://www.mpi.govt.nz/dmsdocument/33708-m-bovis-2019-national-plan-summary

Ministry for Primary Industries. (2019b). *Report of the Mycoplasma bovis technical advisory group (TAG) in response to the terms of reference (June 2019)*. https://www.mpi.govt.nz/dmsdocument/37754/direct

Miranda-Morales, R. E., Rojas-Trejo, V., Segura-Candelas, R., Carrillo-Casas, E. M., Sánchez-Gonzalez, M. G., Castor, R. S., & Trigo-Tavera, F. J. (2008). Prevalence of pathogens associated with bovine mastitis in bulk tank milk in Mexico. *Annals of the New York Academy of Sciences, 1149*(1), 300-302.

Morton, J., Malmo, J., House, J., Mein, G., Izzo, M., & Penry, J. (2014). Mycoplasma bovis in Australian dairy herds. *Australian Veterinary Journal, 92*(9), 322-323. https://doi.org/10.1111/avj.12243

Mulongo, M., Prysliak, T., & Perez-Casal, J. (2013). Vaccination of feedlot cattle with extracts and membrane fractions from two Mycoplasma bovis isolates results in strong humoral immune responses but does not protect against an experimental challenge. *Vaccine, 31*(10), 1406-1412.

Mulongo, M., Prysliak, T., Scruten, E., Napper, S., & Perez-Casal, J. (2013). In vitro infection of bovine monocytes with Mycoplasma bovis delays apoptosis and suppresses production of IFN-γ and TNF-α but not IL-10. *Infection and immunity*.

Murai, K., & Higuchi, H. (2019). Prevalence and risk factors of Mycoplasma bovis infection in dairy farms in northern Japan. *Research in veterinary science, 123*, 29-31. https://doi.org/10.1016/j.rvsc.2018.12.006

Murai, K., Lehenbauer, T. W., Champagne, J. D., Glenn, K., & Aly, S. S. (2014). Cost-effectiveness of diagnostic strategies using quantitative real-time PCR and bacterial culture to identify contagious mastitis cases in large dairy herds. *Preventive veterinary medicine, 113*(4), 522-535.

Murdough, P., Deitz, K., & Pankey, J. (1996). Effects of freezing on the viability of nine pathogens from quarters with subclinical mastitis. *Journal of Dairy Science, 79*(2), 334-336.

New Zealand Government. (2018). *Plan to Eradicate Mycoplasma bovis*. https://www.beehive.govt.nz/release/plan-eradicate-mycoplasma-bovis

Nicholas, R., Ayling, R., & McAuliffe, L. (2008). *Mycoplasma diseases of ruminants*. CABI Wallingford. https://doi.org/https://doi.org/10.1079/9780851990125.0000

Nicholas, R., Fox, L., & Lysnyansky, I. (2016). Mycoplasma mastitis in cattle: To cull or not to cull. *Veterinary Journal, 216*, 142-147. https://doi.org/10.1016/j.tvjl.2016.08.001

Nicholas, R. A., Ayling, R. D., & Stipkovits, L. P. (2002). An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings. *Vaccine, 20*(29-30), 3569-3575.

Nicholas, R. A. J. (2011). Bovine mycoplasmosis: silent and deadly. *Veterinary Record, 168*(17), 459-462.

Nicholas, R. A. J., & Ayling, R. D. (2003). Mycoplasma bovis: disease, diagnosis, and control. *Research in veterinary science, 74*(2), 105-112, Article Pii s0034-5288(02)00155-8. https://doi.org/10.1016/s0034-5288(02)00155-8

Nielsen, L. R., van den Borne, B., & van Schaik, G. (2007). Salmonella Dublin infection in young dairy calves: transmission parameters estimated from field data and an SIR-model. *Preventive veterinary medicine, 79*(1), 46-58.

Nielsen, P. K., Petersen, M. B., Nielsen, L. R., Halasa, T., & Toft, N. (2015). Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of Mycoplasma bovis. *Prev Vet Med, 121*(3-4), 338-342. https://doi.org/10.1016/j.prevetmed.2015.08.009

Oliver, S., Gonzalez, R., Hogan, J., Jayarao, B., & Owens, W. (2004). Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. *National Mastitis Council, Verona, WI*.

Orchard, S. S., & Goodrich-Blair, H. (2004). Identification and functional characterization of a Xenorhabdus nematophila oligopeptide permease. *Appl. Environ. Microbiol., 70*(9), 5621-5627.

Otter, A., Wright, T., Leonard, D., Richardson, M., & Ayling, R. (2015). Mycoplasma bovis mastitis in dry dairy cows. *Veterinary Record, 177*(23), 601-602. https://doi.org/10.1136/vr.h6663

Palmer, M. V., Thacker, T. C., & Waters, W. R. (2009). Histology, immunohistochemistry and ultrastructure of the bovine palatine tonsil with special emphasis on reticular epithelium. *Veterinary Immunology and Immunopathology, 127*(3), 277-285. https://doi.org/https://doi.org/10.1016/j.vetimm.2008.10.336

Paradis, M.-È., Haine, D., Gillespie, B., Oliver, S., Messier, S., Comeau, J., & Scholl, D. (2012). Bayesian estimation of the diagnostic accuracy of a multiplex real-time PCR assay and bacteriological culture for 4 common bovine intramammary pathogens. *Journal of Dairy Science, 95*(11), 6436-6448.

Parker, A., House, J., Hazelton, M., Bosward, K., Morton, J., & Sheehy, P. (2017). Bulk tank milk antibody ELISA as a biosecurity tool for detecting dairy herds with past exposure to Mycoplasma bovis. *J Dairy Sci, 100*(10), 8296-8309. https://doi.org/10.3168/jds.2016-12468

Parker, A., House, J., Hazelton, M., Bosward, K., & Sheehy, P. (2017). Comparison of culture and a multiplex probe PCR for identifying Mycoplasma species in bovine milk, semen and swab samples. *PLoS One, 12*(3).

Parker, A., Sheehy, P., Hazelton, M., Bosward, K., & House, J. (2018). A review of mycoplasma diagnostics in cattle. *Journal of Veterinary Internal Medicine, 32*(3), 1241-1252.

Passchyn, P., Piepers, S., De Meulemeester, L., Boyen, F., Haesebrouck, F., & De Vliegher, S. (2012). Between-herd prevalence of Mycoplasma bovis in bulk milk in Flanders, Belgium. *Research in veterinary science, 92*(2), 219-220.

Penry, J., Malmo, J., Mein, G., & Morton, J. (2014). Molecular testing of Milk: Interpretation and application in Australian dairy herds. Proc. National Mastitis Council 54th Annual Meeting. NMC, Fort Worth, TX,

Petersen, M. B., Krogh, K., & Nielsen, L. R. (2016). Factors associated with variation in bulk tank milk Mycoplasma bovis antibody-ELISA results in dairy herds. *Journal of Dairy Science, 99*(5), 3815-3823.

Petersen, M. B., Pedersen, J., Holm, D. L., Denwood, M., & Nielsen, L. R. (2018). A longitudinal observational study of the dynamics of Mycoplasma bovis antibodies in naturally exposed and diseased dairy cows. *Journal of Dairy Science, 101*(8), 7383-7396.

Petersen, M. B., Pedersen, L., Pedersen, L. M., & Nielsen, L. R. (2020). Field Experience of Antibody Testing against Mycoplasma bovis in Adult Cows in Commercial Danish Dairy Cattle Herds. *Pathogens, 9*(8), 637.

Pfutzner, H. (1990). Epizootiology of the Mycoplasma bovis infection of cattle. *Zentralblatt für Bakteriologie, 20*, 394-399.

Pfützner, H., & Sachse, K. (1996). Mycoplasma bovis as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. *Revue Scientifique et Technique-Office International des Epizooties, 15*(4), 1477-1494.

Pharo, H. (2018). Mycoplasma bovis - review of the diease and evidence from NZ. Proceedings of the Society of Dairy Cattle Veterinarians of the NZVA Annual Conference,

Pinho, L., Thompson, G., Machado, M., & Carvalheira, J. (2013). Management practices associated with the bulk tank milk prevalence of Mycoplasma spp. in dairy herds in Northwestern Portugal. *Preventive veterinary medicine, 108*(1), 21-27.

Pinho, L., Thompson, G., Rosenbusch, R., & Carvalheira, J. (2012). Genotyping of Mycoplasma bovis isolates using multiple-locus variable-number tandem-repeat analysis. *Journal of microbiological methods, 88*(3), 377-385.

Pinnow, C., Butler, J., Sachse, K., Hotzel, H., Timms, L., & Rosenbusch, R. (2001). Detection of Mycoplasma bovis in preservative-treated field milk samples. *Journal of Dairy Science, 84*(7), 1640-1645.

Pothmann, H., Spergser, J., Elmer, J., Prunner, I., Iwersen, M., Klein-Joebstl, D., & Drillich, M. (2015). Severe Mycoplasma bovis outbreak in an Austrian dairy herd. *Journal of Veterinary Diagnostic Investigation, 27*(6), 777-783. https://doi.org/10.1177/1040638715603088

Punyapornwithaya, V., Fox, L., Hancock, D., Gay, J., & Alldredge, J. (2010). Association between an outbreak strain causing Mycoplasma bovis mastitis and its asymptomatic carriage in the herd: a case study from Idaho, USA. *Preventive veterinary medicine, 93*(1), 66-70.

Punyapornwithaya, V., Fox, L., Hancock, D., Gay, J., Wenz, J., & Alldredge, J. (2011). Incidence and transmission of Mycoplasma bovis mastitis in Holstein dairy cows in a hospital pen: A case study. *Preventive veterinary medicine, 98*(1), 74-78.

Punyapornwithaya, V., Fox, L. K., Hancock, D. D., Gay, J. M., & Alldredge, J. R. (2012). Time to clearance of mycoplasma mastitis: the effect of management factors including milking time hygiene and preferential culling. *The Canadian Veterinary Journal, 53*(10), 1119-1122.

Radaelli, E., Castiglioni, V., Losa, M., Benedetti, V., Piccinini, R., Nicholas, R. A. J., Scanziani, E., & Luini, M. (2011). Outbreak of bovine clinical mastitis caused by Mycoplasma bovis in a North Italian herd. *Research in veterinary science, 91*(2), 251-253. https://doi.org/10.1016/j.rvsc.2011.01.006

Radostits, O., Gay, C., Blood, D., & Hinchcliff, K. (2000). Clinical examination and making a diagnosis. In *Veterinary Medicine. A textbook of the Diseases of Cattle, Sheep, Pigs Goats and Horses* (9th ed., pp. 3-39). W B Saunders Company Ltd.

Razin, S. (1999). Adherence of pathogenic mycoplasmas to host cells. *Bioscience reports, 19*(5), 367-372.

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev., 62*(4), 1094-1156.

Register, K. B., Woodbury, M. R., Davies, J. L., Trujillo, J. D., Perez-Casal, J., Burrage, P. H., Clark, E. G., & Windeyer, M. C. (2013). Systemic mycoplasmosis with dystocia and abortion in a North American bison (Bison bison) herd. *Journal of Veterinary Diagnostic Investigation, 25*(4), 541-545.

Reiczigel, J., Földi, J., & Ózsvári, L. (2010). Exact confidence limits for prevalence of a disease with an imperfect diagnostic test. *Epidemiology & Infection, 138*(11), 1674-1678.
Robino, P., Alberti, A., Pittau, M., Chessa, B., Miciletta, M., Nebbia, P., Le Grand, D., & Rosati, S. (2005). Genetic and antigenic characterization of the surface lipoprotein P48 of Mycoplasma bovis. *Veterinary Microbiology, 109*(3-4), 201-209.
Rodriguez, F., Bryson, D., Ball, H., & Forster, F. (1996). Pathological and immunohistochemical studies of natural and experimental Mycoplasma bovis pneumonia in calves. *Journal of comparative pathology, 115*(2), 151-162.
Rogan, W. J., & Gladen, B. (1978). Estimating prevalence from the results of a screening test. *American journal of epidemiology, 107*(1), 71-76.
Romváry, J., Rózsa, J., Stipkovits, L., & Mészáros, J. (1977). Incidence of diseases due to Mycoplasma bovis in a cattle herd. I. Pneumo-arthritis syndrome in calves. *Acta Veterinaria Academiae Scientiarum Hungaricae, 27*(1-2), 29-37.
Rosales, R., Churchward, C., Schnee, C., Sachse, K., Lysnyansky, I., Catania, S., Iob, L., Ayling, R., & Nicholas, R. (2015). Global multilocus sequence typing analysis of Mycoplasma bovis isolates reveals two main population clusters. *Journal of clinical microbiology, 53*(3), 789-794.
Rosati, S., Pozzi, S., Robino, P., Montinaro, B., Conti, A., Fadda, M., & Pittau, M. (1999). P48 major surface antigen of Mycoplasma agalactiae is homologous to a malp product of Mycoplasma fermentans and belongs to a selected family of bacterial lipoproteins. *Infection and immunity, 67*(11), 6213-6216.
Rosenbusch, R. (1985). Bovine pinkeye etiology and pathogenesis. *Bovine Pract, 20*, 150-152.
Rosenbusch, R. (1994). Biology and taxonomy of the mycoplasmas. In *Mycoplasmosis in animals: Laboratory diagnosis* (pp. 3-11). Iowa State University Press.
Rosenbusch, R. (2005). Transmission of Mycoplasma bovis and the syndromes that result in beef and dairy cattle. *Proceedings. 38th Annu Meet Am Assoc Bovine Pract, 38*, 1-5.
Rosengarten, R., & Citti, C. (1999). *The role of ruminant mycoplasmas in systemic infection* (L. Stipkovits, Rosengarten, R., Frey, J., Ed. Vol. 3). European Commission, Brussels.
Rossetti, B. C., Frey, J., & Pilo, P. (2010). Direct detection of Mycoplasma bovis in milk and tissue samples by real-time PCR. *Molecular and cellular probes, 24*(5), 321-323.
Rottem, S. (2003). Interaction of mycoplasmas with host cells. *Physiological reviews, 83*(2), 417-432.
Royster, E., & Wagner, S. (2015). Treatment of mastitis in cattle. *Veterinary Clinics: Food Animal Practice, 31*(1), 17-46.
Ruffo, G., Nani, S., & Astori, E. (1974). Further investigations on the survival of Mycoplasma agalactiae var. bovis. *Bollettino dell'Istituto sieroterapico milanese, 50*(1), 38-40.
Ruffo, G., Nani, S., & Podesta, A. (1969). Survival of Mycoplasma agalactiae var. bovis in several materials and at different temperatures. *Archivio veterinario italiano*.
Ruhnke, H. (1994). *Mycoplasmas associated with bovine genital tract infections* (H. W. Whitford, Rosenbusch, R.F., Lauerman, L.H., Ed.). Iowa State University Press.
Sachse, K., Grajetzki, C., Rosengarten, R., Hänel, I., Heller, M., & Pfützner, H. (1996). Mechanisms and factors involved in Mycoplasma bovis adhesion to host cells. *Zentralblatt für Bakteriologie, 284*(1), 80-92.
Sachse, K., Helbig, J., Lysnyansky, I., Grajetzki, C., Müller, W., Jacobs, E., & Yogev, D. (2000). Epitope mapping of immunogenic and adhesive structures in repetitive domains of Mycoplasma bovis variable surface lipoproteins. *Infection and immunity, 68*(2), 680-687.
Sachse, K., Pfützner, H., Heller, M., & Hänel, I. (1993). Inhibition of Mycoplasma bovis cytadherence by a monoclonal antibody and various carbohydrate substances. *Veterinary Microbiology, 36*(3-4), 307-316.

Sawford, K. (2019). Overview and performance of national milk surveillance for Mycoplasma bovis in New Zealand. Proceedings of the Food Safety, Animal Welfare & Biosecurity, Epidemiology & Animal Health Management, and Industry branches of the NZVA, Wellington.

Schott, C., Cai, H., Parker, L., Bateman, K., & Caswell, J. (2014). Hydrogen peroxide production and free radical-mediated cell stress in Mycoplasma bovis pneumonia. *Journal of comparative pathology, 150*(2-3), 127-137.

Schukken, Y., Smit, J., Grommers, F., Vandegeer, D., & Brand, A. (1989). Effect of freezing on bacteriologic culturing of mastitis milk samples. *Journal of Dairy Science, 72*(7), 1900-1906.

Schukken, Y. H., Wilson, D. J., Welcome, F., Garrison-Tikofsky, L., & Gonzalez, R. N. (2003). Monitoring udder health and milk quality using somatic cell counts. *Veterinary research, 34*(5), 579-596.

Seffner, W., & Pfützner, H. (1980). Mycoplasma mastitis of cattle. 8. Pathological anatomy and histology of experimental Mycoplasma bovis mastitis. *Archiv fur experimentelle Veterinarmedizin, 34*(6), 817-826.

Sergeant, E. (2018). *Epitools Epidemiological Calculators*. Ausvet. http://epitools.ausvet.com.au

Sergeant, E., & Perkins, N. (2015a). Diagnosis and Screening. In C. Makepeace (Ed.), *Epidemiology for Field Veterinarians An Introduction*

(pp. 89-115). CABI.

Sergeant, E., & Perkins, N. (2015b). Investigating Disease Outbreaks. In C. Makepeace (Ed.), *Epidemiology for Field Veterinarians An Introduction* (pp. 26-45). CABI.

Sergeant, E., & Perkins, N. (2015c). Regional Animal Health Programmes. In C. Makepeace (Ed.), *Epidemiology for Field Veterinarians. An Introduction* (pp. 230-246). CABI.

Shahriar, F. M., Clark, E. G., Janzen, E., West, K., & Wobeser, G. (2002). Coinfection with bovine viral diarrhea virus and Mycoplasma bovis in feedlot cattle with chronic pneumonia. *The Canadian Veterinary Journal, 43*(11), 863-868.

Smith, K. L., Todhunter, D., & Schoenberger, P. (1985). Environmental pathogens and intramammary infection during the dry period. *Journal of Dairy Science, 68*(2), 402-417.

Song, Z., Li, Y., Liu, Y., Xin, J., Zou, X., & Sun, W. (2012). α-Enolase, an adhesion-related factor of Mycoplasma bovis. *PLoS One, 7*(6), e38836.

Sordillo, L., Nickerson, S., & Akers, R. (1989). Pathology of Staphyloccus aureus mastitis during lactogenesis: relationships with bovine mammary structure and function. *Journal of Dairy Science, 72*(1), 228-240.

Sordillo, L. M., & Streicher, K. L. (2002). Mammary gland immunity and mastitis susceptibility. *Journal of mammary gland biology and neoplasia, 7*(2), 135-146.

Stalheim, O., & Proctor, S. (1976). Experimentally induced bovine abortion with Mycoplasma agalactiae subsp bovis. *American Journal of Veterinary Research, 37*(8), 879.

Steele, N. M. (2015). *Mastitis pathogen idenitifciation in using polymerase chain reaction in New Zealand milk samples.* Massey University, Palmerston North]. https://mro.massey.ac.nz/bitstream/handle/10179/6892/02

Stipkovits, L., Rady, M., & Glavits, R. (1993). Mycoplasmal arthritis and meningitis in calves. *Acta Veterinaria Hungarica, 41*(1-2), 73-88.

Stipkovits, L., Ripley, P., Varga, J., & Palfi, V. (2000). Clinical study of the disease of calves associated with Mycoplasma bovis infection. *Acta Veterinaria Hungarica, 48*(4), 387-395.

Sugiura, H., & Ichinose, M. (2011). Nitrative stress in inflammatory lung diseases. *Nitric Oxide, 25*(2), 138-144.

Sulyok, K. M., Kreizinger, Z., Fekete, L., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., & Gyuranecz, M. (2014). Phylogeny of Mycoplasma bovisisolates from Hungary based on multi locus sequence typing and multiple-locus variable-number tandem repeat analysis. *BMC veterinary research, 10*(1), 108.

Szacawa, E., Szymańska-Czerwińska, M., Niemczuk, K., Dudek, K., Bednarek, D., & Ayling, R. D. (2016). Comparison of serological, molecular and cultural diagnostic methods for the detection of Mycoplasma bovis infections in cattle. *Animal Science Papers & Reports, 34*(4).

Szklo, M., & Nieto, F. J. (2014). *Epidemiology: beyond the basics*. Jones & Bartlett Publishers.

Tang, J., Hu, M., Lee, S., & Roblin, R. (2000). A polymerase chain reaction based method for detecting Mycoplasma/Acholeplasma contaminants in cell culture. *Journal of microbiological methods, 39*(2), 121-126.

Thermo Fisher Scientific. (2020). *VetMAX™ M. bovis Kit*. https://www.thermofisher.com/order/catalog/product/MPB050#/MPB050

Thomas, A., Dizier, I., Linden, A., Mainil, J., Frey, J., & Vilei, E. (2004). Conservation of the uvrC gene sequence in Mycoplasma bovis and its use in routine PCR diagnosis. *Veterinary Journal, 168*(1), 100-102.

Thomas, A., Leprince, P., Dizier, I., Ball, H., Gevaert, K., Van Damme, J., Mainil, J., & Linden, A. (2005). Identification by two-dimensional electrophoresis of a new adhesin expressed by a low-passaged strain of Mycoplasma bovis. *Research in microbiology, 156*(5-6), 713-718.

Thomas, A., Sachse, K., Dizier, I., Grajetzki, C., Farnir, F., Mainil, J., & Linden, A. (2003). Adherence to various host cell lines of Mycoplasma bovis strains differing in pathogenic and cultural features. *Veterinary Microbiology, 91*(2-3), 101-113.

Thomas, C., Van Ess, P., Wolfgram, L., Riebe, J., Sharp, P., & Schultz, R. (1991). Adherence to bovine neutrophils and suppression of neutrophil chemiluminescence by Mycoplasma bovis. *Veterinary Immunology and Immunopathology, 27*(4), 365-381.

Thomas, C., Willeberg, P., & Jasper, D. (1981). Case-control study of bovine mycoplasmal mastitis in California [Cattle]. *American Journal of Veterinary Research, 42*, 511-515.

Thomas, C. B., Mettler, J., Sharp, P., Jensen-Kostenbader, J., & Schultz, R. D. (1990). Mycoplasma bovis suppression of bovine lymphocyte response to phytohemagglutinin. *Veterinary Immunology and Immunopathology, 26*(2), 143-155.

Timonen, A. A., Katholm, J., Petersen, A., Mõtus, K., & Kalmus, P. (2017). Within-herd prevalence of intramammary infection caused by Mycoplasma bovis and associations between cow udder health, milk yield, and composition. *Journal of Dairy Science, 100*(8), 6554-6561.

Timonen, A. A. E., Autio, T., Pohjanvirta, T., Häkkinen, L., Katholm, J., Petersen, A., Mõtus, K., & Kalmus, P. (2020). Dynamics of the within-herd prevalence of Mycoplasma bovis intramammary infection in endemically infected dairy herds. *Veterinary Microbiology, 242*. https://doi.org/10.1016/j.vetmic.2020.108608

Toft, N., Jørgensen, E., & Højsgaard, S. (2005). Diagnosing diagnostic tests: evaluating the assumptions underlying the estimation of sensitivity and specificity in the absence of a gold standard. *Preventive veterinary medicine, 68*(1), 19-33.

Tolboom, R., Snoep, J., Sampimon, O., Sol, J., & Lam, T. (2008). Mycoplasma mastitis in dairy cattle. *Tijdschrift voor diergeneeskunde, 133*(3), 96-101.

Trinidad, P., Nickerson, S., & Adkinson, R. (1990). Histopathology of staphylococcal mastitis in unbred dairy heifers. *Journal of Dairy Science, 73*(3), 639-647.

Ungureanu, C., Grigore, C., Ionita-Ionescu, F., & Constantinescu, L. (1986). Frequency of mycoplasmae in the semen of reproduction bulls. *Archiv fur experimentelle Veterinarmedizin, 40*(1), 82-87.

USDA. (2008). Prevalence of contagious mastitis pathogens on US dairy operations, 2007. *Fort Collins, Colo: USDA APHIS Veterinary Services Centers for Epidemiology and Animal Health*.

Vähänikkilä, N., Pohjanvirta, T., Haapala, V., Simojoki, H., Soveri, T., Browning, G., Pelkonen, S., Wawegama, N., & Autio, T. (2019). Characterisation of the course of Mycoplasma bovis infection in naturally infected dairy herds. *Veterinary Microbiology, 231*, 107-115.

Van Biervliet, J., Perkins, G. A., Woodie, B., Pelligrini-Massini, A., Divers, T. J., & de Lahunta, A. (2004). Clinical signs, computed tomographic imaging, and management of chronic otitis media/interna in dairy calves. *Journal of Veterinary Internal Medicine, 18*(6), 907-910.

van der Merwe, J., Prysliak, T., & Perez-Casal, J. (2010). Invasion of bovine peripheral blood mononuclear cells and erythrocytes by Mycoplasma bovis. *Infection and immunity, 78*(11), 4570-4578.

van der Molen, E., & Grootenhuis, G. (1979). An investigation of the pathology of Mycoplasma mastitis in the cow. *Vet Q, 1*(3), 126-133. https://doi.org/10.1080/01652176.1979.9693734

Vanden Bush, T. J., & Rosenbusch, R. F. (2002). Mycoplasma bovis induces apoptosis of bovine lymphocytes. *FEMS Immunology & Medical Microbiology, 32*(2), 97-103.

Vanden Bush, T. J., & Rosenbusch, R. F. (2004). Characterization of a lympho-inhibitory peptide produced by Mycoplasma bovis. *Biochem Biophys Res Commun, 315*(2), 336-341. https://doi.org/10.1016/j.bbrc.2004.01.063

Veterinary Council of New Zealand. (2020). *Code of professional conduct for veterinarians*. https://www.vetcouncil.org.nz/Web/Code_of_Professional_Conduct/Code_Of_Conduct

Walz, P. H., Mullaney, T. P., Render, J. A., Walker, R. D., Mosser, T., & Baker, J. C. (1997). Otitis media in preweaned Holstein dairy calves in Michigan due to Mycoplasma bovis. *Journal of Veterinary Diagnostic Investigation, 9*(3), 250-254.

Wawegama, N. K., & Browning, G. F. (2017). Improvements in diagnosis of disease caused by Mycoplasma bovis in cattle. *Animal Production Science, 57*(7), 1482-1487.

Wawegama, N. K., Browning, G. F., Kanci, A., Marenda, M. S., & Markham, P. F. (2014). Development of a recombinant protein-based enzyme-linked immunosorbent assay for diagnosis of Mycoplasma bovis infection in cattle. *Clin. Vaccine Immunol., 21*(2), 196-202.

Wawegama, N. K., Markham, P. F., Kanci, A., Schibrowski, M., Oswin, S., Barnes, T. S., Firestone, S. M., Mahony, T. J., & Browning, G. F. (2016). Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of Mycoplasma bovis infection in feedlot cattle. *Journal of clinical microbiology, 54*(5), 1269-1275.

Wentink, G., Otten, F., Jaartsveld, F., Zeeuwen, A., & Hartman, E. (1987). Mycoplasma bovis arthritis in a herd of dairy cattle. *Tijdschrift voor diergeneeskunde, 112*(13), 795-799.

Wilson, D. J., Goodell, G., Justice-Allen, A., & Smith, S. T. (2009). Herd-level prevalence of Mycoplasma spp mastitis and characteristics of infected dairy herds in Utah as determined by a statewide survey. *Journal of the American Veterinary Medical Association, 235*(6), 749-754.

Wilson, D. J., Justice-Allen, A., Goodell, G., Baldwin, T. J., Skirpstunas, R. T., & Cavender, K. (2011). Risk of Mycoplasma bovis transmission from contaminated sand bedding to naive dairy calves. *Journal of Dairy Science, 94*(3), 1318-1324.

Wilson, D. J., Skirpstunas, R. T., Trujillo, J. D., Cavender, K. B., Bagley, C. V., & Harding, R. L. (2007). Unusual history and initial clinical signs of Mycoplasma bovis mastitis and arthritis in first-lactation cows in a closed commercial dairy herd. *Journal of the American Veterinary Medical Association, 230*(10), 1519-1523.

Wongpakaran, N., Wongpakaran, T., Wedding, D., & Gwet, K. L. (2013). A comparison of Cohen's Kappa and Gwet's AC1 when calculating inter-rater reliability coefficients: a study conducted with personality disorder samples. *BMC medical research methodology, 13*(1), 61.

Wood, S. N., Pya, N., & Säfken, B. (2016). Smoothing parameter and model selection for general smooth models. *Journal of the American Statistical Association, 111*(516), 1548-1563.

Wrathall, A., Ayling, R., & Simmons, H. (2007). Risks of transmitting mycoplasmas by semen and embryo transfer techniques in cattle, sheep, goats and pigs. *CAB reviews: perspectives in agriculture, veterinary science, nutrition and natural resources, 2*(036), 1-31.

Xiao, Y., Bowers, R. G., Clancy, D., & French, N. P. (2005). Understanding the dynamics of Salmonella infections in dairy herds: a modelling approach. *Journal of Theoretical Biology, 233*(2), 159-175.

Zadoks, R. N. (2015). Mycoplasma mastitis - a literature review. *Cattle Practice, 23*, 242-247. <Go to ISI>://WOS:000364897400040

Zhao, G., Zhang, H., Chen, X., Zhu, X., Guo, Y., He, C., Khan, F. A., Chen, Y., Hu, C., & Chen, H. (2017). Mycoplasma bovis NADH oxidase functions as both a NADH oxidizing and O 2 reducing enzyme and an adhesin. *Scientific reports, 7*(1), 1-13.

Zou, X., Li, Y., Wang, Y., Zhou, Y., Liu, Y., & Xin, J. (2013). Molecular cloning and characterization of a surface-localized adhesion protein in Mycoplasma bovis Hubei-1 strain. *PLoS One, 8*(7), e69644.