Epidemiology of *Mycoplasma bovis* in Scottish Dairy Herds

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Abstract

The bacterium *Mycoplasma bovis* (*M. bovis*) causes major economic losses to dairy herds resulting from increased mortality and morbidity, treatment costs, and reduced growth of young stock. There was limited knowledge on the prevalence of *M. bovis* in Scotland and no national monitoring scheme.

Two studies were conducted; a longitudinal bulk tank milk (BTM) prevalence study and a cross-sectional seroprevalence study on dairy calves.

In the longitudinal BTM prevalence study, one hundred and eighty-one dairy herds across Scotland participated in the study which required them to submit four BTM samples roughly three months apart that were tested for the presence of active *M. bovis* infection and for recent exposure. A short questionnaire on general herd management practices were issued to farmers to identify potential risk factors associated with seropositivity. At each of the four sampling points, the proportion of antibody positive herds were 76%, 71%, 83%, and 79%, and overall, 86% of herds tested seropositive in at least one of their four samples. Multivariable logistic regression identified herd history of *M. bovis* as a potential risk factor for the presence of *M. bovis* antibodies. The questionnaire results also provide an updated overview of the common structures and practices on Scottish dairy farms.

Herds were then classified based on the antibody results of their four BTM samples using various methods. Sixty-one percent of herds tested consistently positive for all four samples, 15% consistently negative, and 24% transitional. When classified by k-means clustering of the optical density (OD) trend, the majority of herds had a stable trajectory (44%).

A cross-sectional seroprevalence study was then carried out on a subset of herds from the BTM study (n=36) to determine if there was evidence of exposure to *M. bovis* in youngstock and if there was an association between the BTM and calf seroprevalence. Twenty calves were sampled on each farm (10 animals 4-8 months old and 10 animals 10-14 months old) and a BTM sample collected. There was evidence of youngstock exposure in most herds (58%), and this was associated with the BTM prevalence.

The results of this thesis have demonstrated that *M. bovis* is likely endemic in Scottish dairy herds and has raised further questions on risk factors and within-herd prevalence estimates of *M. bovis*.

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Ireland-Hughes, J., Hotchkiss, E., Young, K., Eze, J., Tongue, S., Mason, C. (2024). '*Mycoplasma bovis* (*M. bovis*) in Scotland – an initial exploration of prevalence, potential risk factors and progression of herd-level disease status over time' [poster, presented by Sue Tongue], *International Symposium on Veterinary Epidemiology and Economics (ISVEE)*, Sydney, Australia, 11-15 November 2024.

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Ireland-Hughes, J., Mason, C., Hotchkiss, E., Tongue, S., Young, K. (2022). 'Buying in cows is associated with the presence of *Mycoplasma bovis* in Scottish dairy herds' [poster presentation], *Society of Veterinary Epidemiology and Preventive Medicine (SVEPM)*, Belfast, UK, 22-26 March 2022.

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Author's Declaration

"I declare that except where explicit reference is made to the contribution of others, this thesis is solely the result of my own work and does not include work presented for another degree at the University of Glasgow or any other institution."

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Abbreviations

AI	Artificial insemination
AIC	Akaike information criterion
AICc	Corrected Akaike Information Criterion
AMR	Antimicrobial resistance
APHA	Animal and Plant Health Agency
BoHV-1	Bovine herpesvirus 1
BRD(C)	Bovine respiratory disease (complex)
BAL	Bronchoalveolar lavage
BRSV	Bovine respiratory syncytial virus
BSE	Bovine spongiform encephalopathy
bTB	Bovine tuberculosis
BTM	Bulk tank milk
BTM study	Bulk tank milk prevalence study
BVD	Bovine viral diarrhoea virus
Calf study	Calf seroprevalence study
C-H	Calinski-Harabasz (criterion)
CHeCS	Cattle Health Certification Standards
CI	Confidence interval

CIA	Critically important antimicrobial
CMS	Composite milk sample
CMT	California milk test
CT	Cycle threshold
DNS	Deep nasopharyngeal swab
ELISA	Enzyme-linked immunosorbent assay
ET	Embryo transfer
EU	European Union
FAO	Food and Agriculture Organisation of the United Nations
GB	Great Britain
HF	Holstein-Friesian
HRP	Horseradish peroxidase
IBR	Infectious bovine rhinotracheitis
IMI	Intramammary infection
IPC	Internal positive control
IQR	Inter quartile range
k	Number of clusters
LKMA	Longitudinal k-means analysis
M. bovis	*Mycoplasma bovis*

n	Number of samples, calves or herds
NZ	New Zealand
OD (%)	Optical density (percent)
OIE	World Organisation for Animal Health
OR	Odds ratio
p	*p*-value, statistical significance
PI3V	Parainfluenza-3 virus
PCR	Polymerase chain reaction
pH	Power of hydrogen
PPLO	Pleuropneumonia-like (agars)
PPV/NPV	Positive/Negative predictive value
RD	Respiratory disease
SCC	Somatic cell count
SDH	Scottish Dairy Hub
Se	Sensitivity
SF	Scottish Farmer
S/P %	Sample-to-positive percentage
Sp	Specificity
SRUC	Scotland's Rural College

TMB	Tetramethylbenzidine
UK	United Kingdom
URT	Upper respiratory tract
USA	United States of America
VIF	Variance inflation factor
VS	Vet Services
WGS	Whole Genome Sequence
WHO	World Health Organisation

Chapter 1 Literature Review

This literature review primarily summarises the knowledge and understanding of *Mycoplasma bovis* and its impact on dairy herds at the time of commencing the PhD (2020-2021). Advancements in any areas that are described in this chapter will be discussed in later chapters, where relevant.

1.1 Etiology and characteristics

The bacterium *Mycoplasma bovis* (*M. bovis*), is a member of the family *Mycoplasmataceae*, in the class *Mollicute*, meaning soft shell, and the genus *Mycoplasma*. Previously named *Mycoplasma agalactae* subsp. *bovis* due to its similarity in etiology and clinical manifestations to mastitis caused by *Mycoplasma agalactae* in sheep and goats, this pathogen is believed to be one of the more infectious species of the *Mycoplasma* genus (Brown et al., 2015). It is one of thirteen *Mycoplasma* species known to infect cattle. *Mycoplasmas* are the smallest and simplest organisms capable of self-replication. They are unable to synthesise peptidoglycan, or its precursors including folic acid, which subsequently synthesises the cell wall. As *Mycoplasmas* are enclosed only in a cell membrane, this makes them susceptible to osmotic pressure, but resistant to the antimicrobials containing glycopeptides and beta-lactams that target features of the cell wall. With the absence of a cell wall, *Mycoplasmas* are pleomorphic in shape and can alter their shape and size in different environments (Hermann, 1992).

1.2 Pathogenesis

M. bovis primarily colonises the mucosal surfaces where it can remain for long periods of time without causing clinical symptoms. The initial site of *M. bovis* colonisation is believed to be the mucosal surfaces of the upper respiratory tract (URT) (Maunsell and Chase, 2019) with subsequent transmission to other sites by crossing mucosal barriers (Hewicker-Trautwein et al., 2002).

Other important sites of *M. bovis* colonisation include the mammary gland, urogenital tract and conjunctiva (Punyapornwithaya et al., 2010). The most significant sites of persistence and shedding are the mammary gland and URT mucosa (Whitford et al., 1994). *M. bovis* is commonly isolated from multiple

sites regardless of the route of contact (Punyapornwithaya et al., 2010). Following oral inoculation with *M. bovis*, colonisation occurred in the tonsils and subsequently spread to the middle ear and lower respiratory tract (Maunsell et al., 2012).

M. bovis is one of the *Mycoplasmas* that forms a biofilm which enables it to hide from the host's defence system (McAuliffe et al., 2006). It may also suppress the immune system, allowing other bacterial and viral pathogens to infect cattle (Nicholas et al., 2008).

1.3 Clinical presentations

The type and severity of clinical symptoms varies between cases of *M. bovis*.

1.3.1 Bovine respiratory disease

The bovine respiratory disease complex (BRDC) is a well-recognised significant health problem in cattle causing substantial effects on the growth, longevity, and reproduction of affected animals (Cernicchiaro et al., 2013). An abundance of microorganisms is naturally present in the upper respiratory tract of animals that exist harmlessly (Bosch et al., 2013). Imbalances in the microbiome can occur and instigate respiratory disease (RD) (Gaeta et al., 2017). Moreover, RD is often multifactorial, and it can be difficult to determine the primary causative agent. The effects of the environment; stress, such as grouping and transportation; weakened immune system and the presence of pathogens, interact together resulting in RD (Kirchhoff et al., 2014). Several pathogens have been associated with respiratory disease. Causative agents include bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI3V) and bovine viral diarrhoea (BVD) virus, bovine herpesvirus 1 (BoHV-1) *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, Arcanobacterium pyogenes, Mycoplasma dispar* and *M. bovis* (Autio et al., 2007; Callan and Garry, 2002; Cirone et al., 2019).

M. bovis is being increasingly recognised as a significant cause or initiating factor of RD (Kusiluka et al., 2000) and is often present early in outbreaks of RD in calves (Arcangioli et al., 2008). Though, the question remains whether *M. bovis* is more often a primary pathogen or an opportunistic secondary pathogen in youngstock. Typically, primary viral pathogens impair host defences by

disrupting host cellular functions and killing infected epithelial cells, consequently increasing the susceptibility of the respiratory tract epithelium, leading to secondary bacterial colonisation (Lima et al., 2016). *M. bovis* can have an immunomodulatory effect on calves predisposing them to other bacterial infections, particularly *Mannheimia haemolytica*, in the respiratory tract (Houghton and Gourlay, 1983). It is likely that *M. bovis* is an initiator of secondary bacterial infections following viral disease. When diagnosing the cause of RD by culture, *M. bovis* is often overlooked due to its specific nutritional requirements and slow rate of growth. Though, the availability of faster diagnostic tools has enabled veterinarians to better detect *M. bovis*.

Various clinical signs have been observed in naturally and experimentally infected animals and are non-specific to infections with *M. bovis* making it difficult to differentiate RD caused by *M. bovis* from that caused by other BRDC pathogens. Acute pneumonia is the commonest disease presentation associated with *M. bovis* in youngstock. Dyspnoea, depression, fever, coughing, rhinorrhoea and anorexia were observed in calves infected with *M. bovis*; these are also common symptoms in calves with other RD pathogens (Adegboye et al., 1996; Autio et al., 2007; Stipkovits et al., 2000). Giovannini et al. (2013) reported a positive correlation of *M. bovis* infections with the presence of pneumonic lesions in young calves. Characteristic disease symptoms associated specifically with *M. bovis* in calves are chronic pneumonia and lameness that fails to respond to antimicrobials. Though these symptoms are also characteristic of bronchiectasis, sequestration, pulmonary abscessation, and infections with *Histophilus somni* (Caswell and Archambault, 2007).

It is not known what age group of cattle are most affected by *M. bovis*, but it appears that calves between 1-4 months of age are particularly affected, presumably due to their underdeveloped immune system (Bennett and Jasper, 1977b; Lamm et al., 2004). Healthy calves with no clinical symptoms are considered reservoirs of *M. bovis* that shed and infect the rest of the herd for months or years (Pfutzner, 1990). Male calves may be at a higher risk of *M. bovis* infection than females which may be caused by feeding lesser quality milk and/or no colostrum to male calves (Lamm et al., 2004).

It is commonplace for individuals within a herd to experience outbreaks of respiratory disease after a period of stress. During a period of stress, the homeostasis of the animal is disrupted, and it is the physiological mechanisms

involved in restoring homeostasis that is referred to as the stress response. Transportation stress is widely recognised as a potential cause of respiratory disease (Earley et al., 2017). Stress during transport is associated with increased severity and incidence of respiratory disease. This is commonly recognised as 'shipping fever' (Duff and Galyean, 2007) and has significant welfare and economic impacts. The prevalence of *M. bovis* was higher on arrival than departure in young cattle transported from France to Italy (Cirone et al., 2019). Other stressors that may lead to respiratory disease include adverse weather conditions, abrupt weaning and castration (Lekeux, 1995), mixing groups and a change of housing.

1.3.2 Mastitis

Mastitis is a significant issue to the dairy industry causing economic losses as a result of reduced milk production and milk quality (Hertl et al., 2014). *M. bovis* is recognised globally as a cause of outbreaks of mastitis in dairy herds (Fox, 2012). It is not commonly reported as a cause of mastitis in the UK, although is probably underreported (GB Veterinary Diagnostic Network, 2023).

Intramammary infection caused by *M. bovis* may be clinical or subclinical. Clinical mycoplasmal mastitis is characterised by a sudden drop in milk production, firm quarters and failure to respond to antimicrobial treatment. Cows at any stage of lactation and dry cows may develop mycoplasmal mastitis (Pfützner and Sachse, 1996). Cows infected with *M. bovis* typically develop subclinical or mild clinical intramammary infections which then develop into chronic mastitis. This can result in chronic mastitis outbreaks within a herd. *M. bovis* can spread between quarters therefore one or more quarters may be affected, resulting in atrophy and secretion of a serous or purulent exudate (Maunsell et al., 2011). Following acute infection, permanent damage to the udder can be severe.

M. bovis-associated mastitis has been described as self-limiting in some instances as the disease diminishes within months of the outbreak and without intervention (Nicholas et al., 2016). Byrne et al. (2005) reported a variation in the persistence of *M. bovis*-mastitis in cows challenged with the pathogen. Within one month of exposure to *M. bovis*, one cow secreted normal milk, while in other cows, the pathogen persisted until the beginning or into their next lactation. Other studies have reported elimination of *M. bovis* infection from

entire herds without intended intervention. As it is not well recorded in the literature, there is little understanding about this feature of a mycoplasma mastitis outbreak.

1.3.3 Arthritis and synovitis

Arthritis is considered a less common symptom of *M. bovis* and is predominantly observed in calves, though all ages of cattle are susceptible. The swelling of tendon sheaths and joints, such as the knee, shoulder, and elbow (Maunsell and Donovan, 2009) has been reported with an accompanying high fever (Hewicker-Trautwein et al., 2002; Stalheim and Page, 1975). Cattle that present with *M. bovis*-associated arthritis typically also have lesions in other organs such as the mammary glands or lungs (Adegboye et al., 1996). Gagea et al. (2006) reported that 47% of pneumonic cases caused in calves by *M. bovis* also had arthritis. Furthermore, *M. bovis* was isolated from the respiratory tracts and synovial fluid of infected calves (Hewicker-Trautwein et al., 2002), demonstrating that *M. bovis* transmits systemically through the bloodstream.

The prevalence of *M. bovis*-induced arthritis among dairy cattle may be underestimated. There is a plethora of factors that contribute to lameness relating to the management, environment, and disease presence (Blowey, 2005). Stress is a suspected trigger of *M. bovis*-associated arthritis and is often apparent following transportation. *M. bovis* may not be a primary suspect when investigating the cause, unless other characteristic symptoms of the disease are observed, thus *M. bovis* may be overlooked by veterinarians.

1.3.4 Otitis Media

Calves infected with *M. bovis* may develop disease of the ear; otitis media (middle), interna (inner) or both; which can cause a unilateral or bilateral ear droop, head tilt, facial paralysis, recumbency and epiphora (Maeda et al., 2003; Walz et al., 1997). Mycoplasmal otitis media is recognised as an increasingly important clinical disease in calves with significant economic impacts (Ayling et al., 2004; Tschopp et al., 2001). The main pathogen isolated from the middle ear of calves with otitis media is *M. bovis* (Francoz et al., 2004; Lamm et al., 2004). Other pathogens isolated from cases of otitis media, interna or both in calves are *Mannheimia haemolytica* (Yeruham et al., 1999), *Arcanobacterium pyogenes* (Baba et al., 1988), *Haemophilus somnus* (McEwen and Hulland, 1985)

and *Pasteurella multocida* (Jensen et al., 1983). Ear disease can occur independently or associated with pneumonia and/or arthritis as reported by Lamm et al. (2004), however the occurrence of mycoplasmal otitis media alone and accompanying other symptoms are not known.

1.3.5 Other clinical signs

Additional, less common symptoms can develop from an infection with *M. bovis* including keratoconjunctivitis, infertility, abortions and brain disease.

Keratoconjunctivitis is the most common and costly eye disease of ruminants. Typically, one eye is infected initially by keratoconjunctivitis, then the other eye becomes infected (Punch and Slatter, 1984). The initial signs of keratoconjunctivitis are photophobia, blepharospasm and increased lacrimation. There are five different clinical presentations of keratoconjunctivitis ranging from mild conjunctivitis and corneal ulceration in acute and subacute disease to ocular rupture and descemetocele formation in the chronic forms (Brown et al., 1998). Rarer symptoms include blindness, severe ulceration, death and panophthalmitis (Bedford, 1976). Whether *M. bovis* is a primary pathogen in keratoconjunctivitis is unclear, though it may occur as a secondary pathogen to *Moraxella bovis* (Nicholas et al., 2008). An outbreak of severe keratoconjunctivitis, believed to be caused by *M. bovis* occurred on a Welsh farm when a group of yearlings were brought to the farm (Kirby and Nicholas, 1996). Clinical symptoms ranged from watery eyes to corneal opacity and transient blindness. In another outbreak of *M. bovis* on an Irish farm, calves developed a range of clinical symptoms including scarring of the cornea and cloudiness of the eye (Ayling et al., 2005).

There is limited knowledge of the impact of *M. bovis* on the reproductive system. Mycoplasma spp. infections were strongly associated with dystocia at last calving, though only *Mycoplasma bovigenitalium* was isolated (Ghanem et al., 2013). Few cases of *M. bovis* are believed to result in reproductive disease, though there is little evidence to support this (Hazelton et al., 2020a, 2018a). Similar to all clinical manifestations of *M. bovis*, the prevalence of *M. bovis*-associated reproductive disease may be significantly underestimated. Infections with *M. bovis* can result in infertility caused by endometrial inflammation (Guo et al., 2014). Experimental inoculation with *M. bovis* has shown some of the potential impacts of the pathogen on the reproductive system. Direct inoculation

of *M. bovis* into the amniotic fluid of pregnant cows resulted in placentitis and induced abortions (Stalheim and Proctor, 1976). Similarly, Kreusel et al. (1989) experimentally infected bulls with *M. bovis* via the urethra or preputium with subsequent infection of the testes. Studies have reported the isolation of *M. bovis* from various sites within aborted foetuses; stomach contents (Byrne et al., 1999); and cerebellum, liver and placenta (Hermeyer et al., 2012). Eaglesome and Garcia (1990) infected semen from a Holstein bull and hamster oocytes with *M. bovis* and reported reduced rates of sperm penetration and fertilisation. Furthermore, *M. bovis* may cause stillbirths. Hermeyer et al. (2012) isolated *M. bovis* from the lungs of a premature calf that died shortly after birth from severe respiratory complications.

Other clinical manifestations of *M. bovis* that are considered rare are diseases of the brain and heart. *M. bovis* was isolated from the brains of calves including one calf that developed neurological signs (Ayling et al., 2005). Examination of the calf at post-mortem revealed a purulent meningitis in which *M. bovis* was the only pathogen isolated. Another calf developed depression, significant weight loss and bruxism. At post-mortem examination of the calf, *M. bovis* was isolated from brain lesions and endocarditis.

1.3.6 Asymptomatic carriers

Some cattle exposed to *M. bovis* do not develop clinical disease and become carriers. These asymptomatic individuals will go undiagnosed at clinical examination but can be detected when tested. The inability to eliminate *M. bovis* from a herd may be influenced by the occurrence of asymptomatic carriers, irrespective of exceptional hygiene practices. Little is known about the nature of this state of infection and few studies have investigated the occurrence of asymptomatic individuals.

Pneumonic lesions were observed in *M. bovis*-positive cattle with no clinical symptoms (Radaelli et al., 2008). Though, the presence of *M. bovis* is not always accompanied by the appearance of visible lesions at necropsy. *M. bovis* was isolated from visibly pneumonic lungs and arthritic joints, as well as from apparently healthy lungs and joints (Gagea et al., 2006).

Previously, *M. bovis* appeared to be predominantly isolated from calves presenting with clinical signs of respiratory disease and was infrequently present

in the respiratory tracts of healthy calves (Thomas et al., 2002a). Now it is known that the prevalence of *M. bovis* among apparently healthy calves is probably underestimated. In another study, herds with recent or current *M. bovis* mastitis had a nasal prevalence of 33.7%, however, no calves exhibited clinical signs typical of respiratory disease (Bennett and Jasper, 1977a).

Asymptomatic cattle may secrete the pathogen from one or multiple sites in the body. *M. bovis* was isolated from the mucosal surfaces of the eyes, nasal cavity and vulvovaginal tract of cattle presenting with no clinical symptoms of *M. bovis* (Punyapornwithaya et al., 2010). Additionally, *M. bovis* was isolated from milk samples from some cows in the herd. The majority of animals within this herd had no clinical symptoms associated with *M. bovis* mastitis, pneumonia, or lameness.

In addition to completely asymptomatic animals, Houlihan et al. (2007) reported mastitic cows showing no clinical signs of mastitis with the exception of a mild mucoid discharge from the udder. It is evident that clinical signs of *M. bovis*-associated disease may be marginal and thus go undetected (Punyapornwithaya et al., 2010).

It is not known exactly how long animals may remain carriers of *M. bovis*. In one study, a single heifer tested positive in repeated nasal swabs collected at weaning, before breeding and post-calving (Hazelton et al., 2020b), highlighting that it is possible that cattle infected when they are young may remain carriers for a long time.

The introduction of asymptomatic carriers to a herd is considered the main route in which a herd becomes infected with *M. bovis*. Accompanied with intermittent shedding of the pathogen, this could be essential for maintenance of disease within a herd.

1.4 Transmission and risk factors

The control of an infectious pathogen relies on an understanding of disease dynamics (Dobson and Meagher, 1996). Various routes of transmission of *M. bovis* are identified, yet further work is required to quantify the importance of the different routes.

The main route of infecting a naïve herd with *M. bovis* is thought to be the introduction of carrier animals (Tschopp et al., 2001). Animal movements and

purchasing introduce the potential for disease transmission, therefore many farms operate a closed herd policy and do not purchase livestock. Transmission may occur at the time of introduction or later due to delayed shedding of the pathogen (Fox et al., 2005). The introduction of a breeding bull into the herd significantly increases the risk of disease entering a naïve herd (Gille et al., 2018).

Direct contact between infected and naïve animals is considered the main route in which *M. bovis* spreads within a herd, particularly in group housing of calves. The movement and purchasing of animals introduce the potential transmission of disease.

1.4.1 Milk and colostrum

Maternal colostrum contains essential nutrients and immunoglobulins, such as IgG which is essential for passive immunity (Morrill et al., 2012). Colostrum protects neonates for the first few weeks and months of life against infectious diseases (Johnson et al., 2007) until their own immune defences are fully developed, though colostrum is one of the initial routes of exposure to pathogens (Godden et al., 2012). Milk and colostrum may be contaminated through poor hygiene practices at collection, feeding, handling and storage (Fecteau et al., 2002). Examples include not cleaning udders before milking or using dirty milking equipment and buckets (Godden et al., 2019). Feeding milk or colostrum from subclinically or clinically infected cows was shown to significantly increase the risk of *M. bovis* colonisation of the nasal tract of recipient calves in one study (Walz et al., 1997). Feeding older calves with California Milk Test (CMT) positive milk was associated with the presence of *M. bovis* in veal calves (Schönecker et al., 2020). This association would be expected as CMT positive milk has a higher somatic cell count (SCC) and thus a higher bacterial load present within the milk.

Transmission of *M. bovis* specifically by colostrum is not well documented. Gille et al. (2020) reported that only 1.9% of colostrum samples (7/368) tested positive for *M. bovis* from herds with recent *M. bovis* infection. Furthermore, those positive samples came from 5 out of the 17 recently infected herds. Although the prevalence was low, the authors highlighted that colostrum was sampled by the farmers and the potential for noncompliance of the set methods

could have influenced the results. This raises the question of whether transmission via colostrum is an important route of spread or whether other routes of transmission are much more important.

Lactating cows infected with *M. bovis* may shed the pathogen intermittently in their milk and colostrum. In a study by Stalheim and Page (1975), two lactating cows each nursing a calf were inoculated experimentally with *M. bovis* (at that time called *M. agalactiae* subsp. *bovis*). After several days, the calves developed swollen knees and subsequent lameness. At post-mortem, *M. bovis* was isolated from synovial fluid from both calves. *M. bovis* has been isolated from bulk tanks where there were issues with mastitis and *M. bovis*-associated clinical symptoms in calves fed cows' milk (Butler et al., 2000; Walz et al., 1997). In two farms there were no opportunities for direct contact between the milking herd and calves, yet there were two coinciding outbreaks of mastitis in cows and pneumonia in young stock (Aebi et al., 2012). The authors stated that the only connection between the two groups was the feeding of cow's milk to calves. Similarly, the nasal prevalence of *M. bovis* among calves was higher in herds where *M. bovis*-mastitic milk was fed to calves (Bennett and Jasper, 1977a).

1.4.2 Breeding and congenital transmission

The introduction of a breeding bull into the herd significantly increases the risk of introducing *M. bovis* into a naïve herd (Gille et al., 2018). Experimental infection of the urethra and preputium of bulls with *M. bovis* resulted in shedding of the pathogen in semen (Kreusel et al., 1989). *M. bovis* was isolated from commercial bull semen in Israel (Amram et al., 2013) and from embryos following fertilisation with *M. bovis*-positive semen (Bielanski et al., 2000). The introduction of *M. bovis* into a naïve herd via infected semen was first demonstrated by Haapala et al. (2018) who introduced *M. bovis* into two Finnish herds free of disease via artificial insemination (AI) with semen from a *M. bovis*-positive bull. This resulted in subsequent outbreaks of mastitis within both herds four weeks later. Similarly, bulls seroconverted after serving cows in three herds with recent or current clinical *M. bovis*-associated symptoms (Hazelton et al., 2018a). These findings suggest that regardless of the method of insemination, whether natural or AI, there is a risk of introducing this pathogen into a herd through breeding.

M. bovis was isolated from embryos fertilised with *M. bovis*-positive semen (Bielanski et al., 2000) though to the authors knowledge, there is no evidence of *M. bovis* transmission to the dam during embryo transfer (ET). Therefore, introducing *M. bovis* to a naïve herd via ET should be unlikely.

There has been little focus on the potential for *M. bovis* to spread from dam to calf during and post-gestation. Calves may become infected *in utero*, at parturition of their dam or other cow in the maternity unit (Hermeyer et al., 2012). Stalheim and Proctor (1976) inoculated the amniotic fluid of pregnant cows with *M. bovis* and subsequently isolated *M. bovis* from several tissues of the foetuses. Similarly, (Hermeyer et al., 2012) infected cows in the third trimester resulting in abortion of one foetus and premature birth of a calf that later died due to respiratory disease. Both foetus and calf tested positive for *M. bovis*. *M. bovis* was isolated from a foetus, however the disease status of the dam was unknown (Byrne et al., 1999). Infection by also be transmitted through vaginal discharges from the dam at the time of calving (Bennett and Jasper, 1977b; González and Wilson, 2003). *M. bovis* is not thought to transfer to the dam during *in vitro* embryo production (Peippo et al., 2020). The occurrence of congenital transmission is not well documented and not believed to be an important route of transmission, though may be disregarded as the cause of abortions and premature deaths.

1.4.3 Fomites and the environment

M. bovis is thought to survive in the environment, on equipment and clothing for months following an infection (Justice-Allen et al., 2010). The formation of a biofilm may contribute to the ability of *M. bovis* to survive in the environment for long periods of time (McAuliffe et al., 2006), yet this feature of the pathogen is poorly understood. Outbreaks within a herd are likely facilitated via transmission on milking and calf feeding equipment.

Feed buckets, tubes and teats may be an important route of transmission between calves. Group feeding of calves enables the spread of *M. bovis* between individuals. Higher numbers of young calves per drinking nipple was associated with the presence of *M. bovis* in the upper respiratory tract of veal calves (Schönecker et al., 2020). *M. bovis* was also cultured from swabs taken from calf housing in an Italian veal farm (Piccinini et al., 2015).

Horizontal transmission via direct contact with infected individuals is a major concern in group housed calves. Pre-weaned calves are generally housed individually and subsequently moved to group housing at, or shortly after, weaning (NAHMS (National Animal Health Monitoring System), 2007). Individually housed calves had reportedly less cases of respiratory disease within the first 90 days than group housed calves (Cobb et al., 2014).

M. bovis could be spread efficiently between cows in the milking parlour via the hands of the milker, the clusters, or from contaminated and improper treatment of teats (González and Wilson, 2003; Jasper, 1979). During and after milking, the opening of the teat canals creates an opportunity for infection to enter the teats (Pfützner and Sachse, 1996). Stimulation until milk let down and fore-stripping were identified as potential risk factors for *M. bovis* presence within a herd (Aebi et al., 2015), as these practices expose the teat to contact with potentially infected milkers' hands.

Other environmental sources include bedding/resting places and calving areas. *M. bovis* reportedly survived in sand bedding from farms infected with *M. bovis* mastitis for eight months (Justice-Allen et al., 2010). Also, herds that did not separate cows at calving into a separate calving pen were at an increased risk of contracting *M. bovis* than those that were separated (Gille et al., 2018). During the periparturient period, cows experience a negative energy balance as a result of metabolic and hormonal fluctuations (Mordak and Anthony, 2015). Subsequently, the immune system is compromised which may increase shedding of *M. bovis* at calving. Additionally, segregating cows during parturition from the main herd could protect them from potential carriers that may be present and limit environmental contamination in calving areas.

1.4.4 Aerosol

Airborne transmission of *M. bovis* is possible (Kanci et al., 2017), particularly in sheds with poor ventilation (González and Wilson, 2003). In a herd where *M. bovis* was isolated from vaginal, nasal and milk samples, (Jasper et al., 1974) isolated *M. bovis* via bacterial culture when the agar plate was exposed to the barn air of one herd, though *M. bovis* was not cultured in three other samples. Successful isolation of *M. bovis* from the air is likely affected by the number of colony-forming units of *M. bovis* in the air, time of sample collection, transport and the presence of other pathogens in the air (Soehnlen et al., 2012).

Furthermore, the specific nutritional requirements for culturing *M. bovis* and the slow rate of growth may also delay the isolation of the pathogen.

1.4.5 Other potential risk factors

Other risk factors that may be associated with the presence of *M. bovis* in a herd are larger herds and increasing the size of a herd (UK Cattle Expert Group, 2018). No associations have been observed between the season and the presence of *M. bovis* infections in cattle.

1.5 Herd-level impacts

It is well established that bovine respiratory disease (BRD) results in reduced growth and production of youngstock, however, there is conflicting evidence as to whether *M. bovis*-associated RD is associated with reduced growth rates in calves. No associations were found between the growth rates of feedlot calves and serostatus in one study (Martin et al., 1990), whereas in another more recent study, seropositive weaned beef calves had lower weight gains than seronegative calves (Hanzlicek et al., 2011).

Most, if not all studies on the associations of *M. bovis* serostatus with calf growth rates were based on beef feedlots in the United States which are vastly different to beef rearing systems in the UK. Thus, the findings are likely not comparable to that of dairy calf rearing systems in the Scotland.

With the exception of beef calves that are later used as breeding bulls, dairy heifer calves have a longer lifespan than beef calves as they will enter the milking herd. The long-term impacts of *M. bovis* on growth and production have not yet been thoroughly investigated. One study reported that there was an association between the *M. bovis* seroprevalence of heifer calves and undesired early departure from the herd, i.e. culled or died (Petersen et al., 2019).

Intramammary infections (IMI) cause high SCCs and spontaneous lipolysis and proteolysis which results in reduced milk fat and urea contents (Larsen et al., 2010; Vidanarachchi et al., 2015; Zhang et al., 2015). The log transformed SCC of *M. bovis*-positive milk was on average 0.8 units higher than negative cows (Timonen et al., 2017). Significantly higher SCC on average were observed in

cows with mycoplasma spp. coinfections (389,320 cell/mL) compared to apparently healthy cows (67,330 cell/mL) (Al-Farha et al., 2017).

Following experimental infection with *M. bovis*, cows within the first 6-8 weeks of lactation experienced a considerable decrease in milk production lasting for 8-12 weeks (Ruhnke et al., 1976). More recently, Pothmann et al. (2015) reported a significant drop in milk production by 60% in an outbreak of *M. bovis* in an Austrian dairy herd. Furthermore, Timonen et al. (2017) reported a reduction of up to 3.0kg daily milk yield in cows that tested positive for *M. bovis*. Al-Farha et al. (2017) reported a reduction in milk yield of 2L/day in cows infected with *M. bovis* compared to healthy cows. This was not statistically significant, though cows with a coinfection of mycoplasmas produced significantly less milk per day (-5.4L/day) than healthy cows. Similarly, cows infected with mycoplasmas and other mastitis pathogens produced yielded significantly less milk (-6.5L/day).

It is apparent that infections with *M. bovis* negatively affect milk production, yet the economic impact of *M. bovis*-associated mastitis is not yet known. Coinfections with other mycoplasmas and/or other conventional mastitis pathogens have greater impacts on the production of milk than infections with only *M. bovis*.

1.6 Diagnosis

The reasons for delayed diagnosis of *M. bovis* infections relate to the widespread lack of awareness and appreciation for the significance of the pathogen. *M. bovis* was considered an opportunistic pathogen and was only recently suggested to be a primary pathogen. Clinical symptoms of *M. bovis* are not pathognomonic, therefore other pathogens remain suspect prior to a correct diagnosis. Respiratory and mastitic clinical manifestations of *M. bovis* are similar to a number of other pathogens, therefore *M. bovis* cannot be accurately diagnosed without laboratory testing.

1.6.1 Culture

Previously, *M. bovis* diagnosis relied heavily upon culture methods. While culturing provides a definitive diagnosis, this approach is laborious and combined with the pathogen's specific nutritional requirements, it is a drawn-out process.

Although cultivation is highly sensitive, the sensitivity can be reduced by a number of factors including: the length of time between culturing and interpretation of results, taking an independent sample (i.e. not sampling over a period of time), not sampling from the known carrying sites of *M. bovis*, and improper storage and handling of samples for testing (Calcutt et al., 2018; Gille et al., 2018; Pfützner and Sachse, 1996).

With a lengthy culturing time, it can be challenging to keep the organism viable for growth, thus the handling and storage of the organism is important to ensure *M. bovis* can be accurately diagnosed (Parker et al., 2017a). Increasing the length of time between culture and interpretation reduces the sensitivity in a number of ways. It is common for *M. bovis* to be involved in co-infections with other pathogens (Justice-Allen et al., 2011; Mehinagic et al., 2019; Szacawa et al., 2015). These other pathogens may be faster growing with less specific nutritional requirements than *M. bovis*, which could result in overgrowth and consequently, those bacteria will be wrongly identified as the cause of infection rather than *M. bovis* (Dudek et al., 2020). Furthermore, with an extended time between culturing and interpretation, there is an increased risk of contamination by other pathogens, which again could result in a false negative diagnosis.

Consequently, a negative result cannot be concluded until 7-10 days following initial culture. Nevertheless, cultures are still used as they are convenient for diagnosing a single animal (Sachse et al., 1993). *M. bovis* is often accompanied by other pathogens, i.e. pathogens involved in the bovine respiratory disease complex or other mastitic pathogens. Faster growing and less nutritionally demanding pathogens may colonise first and result in an incorrect diagnosis (Olde Riekerink et al., 2006).

Cultivation of *Mycoplasma* spp. requires complex media due to their simplistic structure and limited ability to synthesise macromolecules essential for growth, including fatty acids and amino acids (Parker et al., 2018). Mycoplasma media is typically highly enriched with beef heart infusion, yeast extract, peptone, serum and other supplements (McVey et al., 2013). Specific pleuropneumonia-like (PPLO) agars are commonly used for cultivation of *M. bovis* with selective antimicrobials such as antibiotics or thallium acetate in order to prevent overgrowth of other bacteria.

Mycoplasma spp. growth is extremely susceptible to any pH changes, with a pH below 6.5 limiting growth and initiating cell death, and a pH greater than 8.0 also resulting in death of the cells (Nicholas et al., 2008). Due to the restricted pH range for mycoplasma spp. growth, the medium should be well buffered. As mycoplasmas are devoid of a cell wall, there is an increased risk of cell lysis in hypo-osmotic media. To increase the tonicity of the culture medium, sodium chloride is required.

Previously, it was recommended that inoculated culture media should be incubated at 37°C and 10% CO_2 for 7-10 days (Hale et al., 1962; Middleton, Fox and Pighetti, 2017). Lowe et al. (2018) then suggested that the CO_2 conditions for culturing *M. bovis* may be less restricted than previously thought. More recently, Biesheuvel et al. (2024a) reported that despite lower growth of *M. bovis* cultured in ambient air during the first week, there was no difference in the detection of *M. bovis* growth after ten days when cultured in ambient air, 5% CO_2, or 10% CO_2, confirming that *M. bovis* culture does not strictly require supplemental CO_2.

Colonies of *M. bovis* have a typical 'fried egg' appearance, with denser growth of the colony in the centre and within the medium, whereas surrounding growth occurs on the surface (González and Wilson, 2003).

Ultimately the shift from culture to PCR was driven by the fact that it is imperative to the farmer that *M. bovis* is diagnosed quickly. This pathogen is highly contagious within a herd, therefore delaying the diagnosis allows for more transmission within the herd (Parker et al., 2017a). If *M. bovis* is diagnosed, the infected animals can be removed and prevent further transmission.

1.6.2 Detection of DNA

The development of polymerase chain reaction (PCR) tests has enabled faster diagnosis of *M. bovis* infections with high sensitivity and specificity (Wisselink et al., 2019). These tests are used for the detection of *M. bovis* by amplification of DNA. PCR is a more expensive diagnostic tool compared to culture methods, therefore samples may be pooled (Murai et al., 2014). As well as diagnosing *M. bovis* alone, multiplex PCRs have been developed that detect multiple mycoplasma species (Gioia et al., 2016).

Real time PCR has the added benefit of quantifying the amount of *M. bovis* DNA in the sample, however validation is important to determine a cut-off of cycles so that late amplification is not classified as a positive result. Late amplification of *M. bovis* DNA may occur in samples containing low volumes of the target DNA, sample contamination, cross-amplification of other pathogens with the same DNA, the presence of PCR inhibitors, or in samples with degraded DNA (Cai et al., 2005; Mouliou and Gourgoulianis, 2021; Toohey-Kurth et al., 2020).

Where culture methods can only detect viable organisms, one of the benefits of using PCR is that it can also detect non-viable (i.e. dead) organisms (Parker et al., 2018). Though this can produce a false positive result (Wolffs, Norling and Radstrom, 2005). In terms of disease detection within a herd, using any diagnostic test, it is imperative to reduce the number of false negative results. When testing for a very virulent disease or if looking to eliminate a disease from a herd, it may be more important to reduce the number of false negatives.

It is not possible to say how long DNA lasts post-infection, and it will vary in different conditions, however, the detection of non-viable organisms is evidence that there has been a recent infection. Therefore, although PCR testing can produce false positives due to non-viable organisms, at the least, it gives a good indication of whether the pathogen in question has recently been present, and the farmer and vet can take action to monitor and treat animals (Gioia et al., 2016).

Intermittent shedding of *M. bovis* in chronically infected cows was reported by (Biddle et al., 2003). This may be ongoing for months or years (Punyapornwithaya et al., 2010), and there is yet to be an explanation for this feature of the disease. This feature of *M. bovis* creates a challenge to accurately detect the pathogen as sampling in the absence of shedding will give a false negative result. Moreover, asymptomatic carrier animals may also shed the pathogen intermittently, creating an even bigger challenge when trying to detect reservoir animals within the herd. This is why repeated sampling is important to try and capture these infectious individuals.

At the time of commencing this thesis, there was no research into the genetic diversity of *M. bovis* in Scotland, nor the UK. Recently, a study sought to Whole Genome Sequence (WGS) strains of *M. bovis* in Ireland and Scotland (McAloon et al., 2025). The results indicated that the strains in Scotland and Ireland may be genetically similar to strains in Europe. Furthermore, there appeared to be no

clear indication of specific clinical symptoms being linked to particular WGS strains.

Molecular studies seeking to genotype *M. bovis* are ongoing. Whole genome sequence strains of *M. bovis* in Ireland and Scotland may be genetically similar to strains in Europe (C. Mason, personal communication).

1.6.3 Detection of antibodies

Antibodies are detectable for many months post-infection, whereas the detection of active infection relies upon the animal being actively infected at the time of sampling (Nicholas and Ayling, 2003). This makes the detection of antibodies a more practical outcome measure for research studies as they are easier to capture. Furthermore, antibody testing is quick and inexpensive (Petersen et al., 2020).

There are a range of different serological tests available, however indirect enzyme-linked immunosorbent assays (ELISAs) are the tests most commonly used for routine herd diagnostics (Parker et al., 2018). ELISA tests can be used to detect antibodies in individual animals (e.g. in milk, synovial fluid, nasal swabs and blood), in pooled milk samples, and in the bulk tank milk (BTM).

Different ELISAs with varying sensitivities and specificities are commercially available for the detection of *M. bovis* antibodies. The sensitivity and specificity of these ELISAs can vary greatly, but according to the manufacturers, they are close to 100%. When used under field conditions, and following optimisation of the test cut-offs, the sensitivity and specificity of these tests is reportedly lower. At the time of commencing this PhD, there was the ID Screen® *Mycoplasma bovis* Indirect ELISA (Grabels, France), and various Bio-X ELISAs (Bio-X Diagnostics S.A., Rochefort, Belgium). The ID Screen indirect ELISA has the highest reported sensitivity compared to the Bio-X ELISAs (Petersen et al., 2020).

The sensitivity and specificity of these diagnostic tests will differ when testing individual cow milk compared to testing BTM or pooled samples. When testing samples from individual animals, the test sensitivity and specificity will generally be higher as the sample only represents the antibody levels in that individual cow. When testing BTM samples, the antibodies from each cow are diluted which could lead to a negative result, when there are in fact antibody positive cows contributing to the BTM. Also, even if there is a small proportion

of antibody positive cows in the herd, if they are producing higher volumes of antibodies, the result could be positive, or even a high positive, which would suggest that a large number of cows are producing antibodies. Consequently, the sensitivity of a diagnostic test may be lower when testing a BTM or pooled sample.

There are other diagnostic tests available to detect antibodies to *M. bovis* such as agglutination tests (Gagea et al., 2006), however ELISAs are more commonly used as they have a higher sensitivity.

The duration of antibody detection post-infection is not well documented (Nicholas and Ayling, 2003). Byrne et al. (2000) reported antibody detection in individual samples of cows' milk up to 20 weeks post-infection. More recently, Petersen et al. (2018a) demonstrated that the antibody dynamics in individual animals can be highly variable. Some cows with clinically diagnosed mastitis may have detectable antibody levels for months following infection, whereas antibodies may only be detectable for a matter of weeks in others. Also, in a Finnish study, *M. bovis* antibodies were still detected for one year in dairy herds, even in the absence of clinical *M. bovis* mastitis (Vähänikkilä et al., 2019). In these herds, the proportion of antibody positive youngstock began to decrease in subsequent visits when *M. bovis* was not detected. Furthermore, seroconversion can take up to three weeks, meaning that if cattle are tested before seroconversion, there will be no antibodies to detect (Wawegama et al., 2014). It appears that antibodies are generally detectable for long periods of time following an active infection.

1.6.4 Sampling

Milk

Sampling from the BTM can provide a herd-level diagnosis of *M. bovis* and indicates that at least one cow within the herd has an infection. It is not known exactly what proportion of positive cows (active infection or antibody positive) must be contributing to the tank to obtain a positive test result. A limitation with this method is that clinically infected cows with current infections typically do not contribute to the bulk tank if the animal is receiving antimicrobial treatment or produced abnormal milk. This affects diagnosing the presence of the pathogen itself however if the infection has been circulating in the herd for

a period of time, then antibodies will be present throughout the herd as they are shed for many months after an infection (Nicholas and Ayling, 2003). Furthermore, due to the dilution of pathogens (or antibodies), the tests may produce false negatives if not enough infected (or recently infected) individuals are contributing to the BTM, which is why repeated sampling of the BTM is more favourable rather than sampling once. BTM sampling can be used as an initial tool to detect disease or antibodies at herd level prior to individual cow milk sampling.

Animals with possible subclinical intramammary infections caused by *M. bovis* can be identified with high SCCs (Fox et al., 2005). Some cows with *M. bovis*-mastitis had low SCCs <200,000 cells/ml, although they may have been in the early stages of infection (Higuchi et al., 2013).

Composite milk samples (CMS) may be used as a quicker and cheaper alternative to individual quarter sampling. The sensitivity of CMS for the identification of mycoplasma mastitis has varied. *Mycoplasma* spp. were isolated from 54% of CMS and 39% of quarter milk samples (QMS) taken from 10 cows over the course of 28 days (Biddle et al., 2003), suggesting comparable detection rates between both sampling methods. Conversely, 3 out of 15 infected cows had a negative CMS result. In another study, there was a higher proportion of cows infected with mycoplasma mastitis in only one quarter than cows with more than one infected quarter (Pinho et al., 2013).

Nasal swabs

Infections with *M. bovis* are also detected by collecting nasal swabs from youngstock. Nasal swabs are a quick and affordable method of diagnosing youngstock, however, *M. bovis* may only colonise tonsils and therefore not be sampled from the shallow nasal swabs (Maunsell et al., 2012). To ensure that deeper infection is not missed, the pharyngeal lymphoid tissue can be sampled by collecting a deep nasopharyngeal swab (DNS), though this is a more expensive and less practical approach (Pohjanvirta et al., 2021). One of the drawbacks to nasal swabbing, particularly deeper swabs is the risk of contamination in the nasopharyngeal passage (Thomas et al., 2002b).

Alternatively, bronchoalveolar lavage (BAL) samples can be collected. This involves passing a BAL catheter through the nasal passage into the lower

respiratory tract (Davidson et al., 2020) with or without endoscopic guidance. Depending on the BAL catheter used, there is increased risk of contamination by organisms in the nasal cavity (Allen et al., 1991). Van Driessche et al. (2017) found no difference in the detection of *M. bovis* via BAL or DNS in calves with RD or healthy calves. Similarly, Pohjanvirta et al. (2021) reported an agreement of 91% between BAL and DNS.

Blood

Sampling from blood is also used for detecting active *M. bovis* infections and antibodies (Arcangioli et al., 2008). Serum sampling of youngstock and older cattle is often incorporated into herd screening for various diseases (Booth and Brownlie, 2016; Kalis, 2003). This method of disease detection can be costly to a farmer as the samples must be collected by a vet, unlike milk samples or nasal swabs (UK Veterinary Surgeon's Act 1966). Although testing of serum for the detection of *M. bovis* is common practice in veterinary medicine, it is still unclear what the best approach is to detect *M. bovis* in a herd, i.e. what groups of animals should be targeted.

All ages of cattle can be blood sampled, however antibodies detected in calves under six months of age may be maternally derived as the immune system develops up until this age (Butler et al., 2006; Chase, Hurley and Reber, 2008). Petersen et al. (2018b) conducted a study following the antibody levels in calves in four herds with recent *M. bovis* infection using two different ELISA tests (Bio K302, BioX Diagnostics, Rochefort, Belgium; in-house indirect IgG ELISA, MilA ELISA). The authors reported that the BioX ELISA was unable to detect *M. bovis* antibodies in calves under three months of age whereas the MilA ELISA could detect antibodies in calves under three weeks of age. This highlights the importance of carefully interpreting antibody results in young calves and also to select an ELISA that will detect antibodies in young calves.

The authors noted that the relationship between age and the estimated antibody units measured by the MilA ELISA was similar in three herds. There was an initial increase, then a plateau followed by a decrease. The peak in antibody units ranged between 60 and 120 days of age.

What is interesting is that in those three herds, the main group of animals infected, and the main clinical signs all differed, i.e. cows and or calves affected, and clinical symptoms in calves was a combination of pneumonia, otitis media or arthritis. Despite these differences among herds, the antibody levels followed a similar trend. This suggests that regardless of the clinical disease in the calves, the antibodies appear to be present for a similar duration. More research is required to fully understand this trend and determine how long antibodies are present following infections with *M. bovis*.

Other samples

Samples of the synovial fluid may be collected for the detection of *M. bovis*-associated lameness (Hewicker-Trautwein et al., 2002). Furthermore, swabs of the conjunctiva may be taken to detect *M. bovis*-associated keratoconjunctivitis (Alberti et al., 2006).

1.7 Treatment

One of the major concerns with *M. bovis* is the lack of available, effective treatment options. Due to the unique structure and action of *M. bovis*, many antimicrobial groups are ineffective against the pathogen. Sulfonamide antimicrobials target the synthesis of folic acid, however *M. bovis* does not synthesise folic acid. Additionally, *M. bovis* lacks a cell wall, therefore beta-lactam antimicrobials are ineffective as they target the cell wall. As a result, there is an increased reliance on Macrolides, Fluoroquinolones, Lincosamides and Tetracyclines that target protein or DNA synthesis (Maunsell et al., 2011). A number of antimicrobials within these classifications are critically important antimicrobials (CIA) (European Medicines Agency, 2020). The CIAs were classified by the World Health Organization (WHO), the Food and Agriculture Organisation of the United Nations (FAO), and the World Organisation for Animal Health (OIE) in 2003. Antimicrobials were categorised based on their importance to human health and impose restrictions on the use of specific antimicrobial groups. As well as this global effort to reduce the use of antimicrobials in veterinary medicine, UK policies are in place urging farmers to minimise their prophylactic and metaphylactic antimicrobial usage and instead utilise vaccines (Gov, 2018). Thus, there is a greater focus on control and prevention of *M. bovis*.

1.8 Control and prevention

Good biosecurity is imperative for the maintenance of disease-free farms. Purchasing cattle increases the risk of introducing infected cattle into a naïve herd. Likewise, naïve cattle could be introduced into an endemically infected herd. The ability to know the disease status of purchased cattle is very difficult, which is why when introducing new animals to a herd, it is recommended to isolate the new animals for a minimum of one month to allow time for health checks to be conducted (Carr and Howells, 2018). This length of time is not always practical but should be aimed for. A study in Belgium indicated that housing recently purchased cattle in the same airspace as the main herd was a risk factor for a positive *M. bovis* PCR test of pooled non-endoscopic bronchoalveolar lavage samples (Pardon et al., 2020). One of the most effective approaches to minimise the risk of disease introduction is to operate a closed herd policy, though this could have an impact on the introduction of superior genetics to the herd (Robertson, 2020).

As previously discussed, there is the potential for *M. bovis* to be transmitted via semen from infected bulls, however no semen banks currently test for the presence of *M. bovis*.

For prevention of microbial contamination of semen intended for use in artificial insemination, it must be treated with the following antimicrobials: Streptomycin, Penicillin, Lincomycin and Spectinomycin, with the option of an alternative combination against leptospires, mycoplasmas and campylobacters, The Bovine Semen (Scotland) Regulations 2007 (SSI/2007/330).

Following the detection of *M. bovis* in New Zealand in 2017, a national eradication scheme was implemented, requiring all farms throughout the country to partake in monthly BTM testing, testing of individual animals, movement restrictions, decontamination of infected premises, and culling (Laven, 2019).

1.8.1 Vaccination

Another effective method for preventing the introduction of disease into a herd is to use vaccines. Prior to the start of the PhD project, there was no licenced vaccine available for use against *M. bovis* in the UK, therefore the only option

was to use autogenous vaccines. These are herd specific as they are created using a sample of the specific *M. bovis* strain that is present in the herd. Autogenous vaccines are also expensive therefore their use is minimal.

At the time of commencing this thesis, there was only one commercial *M. bovis* vaccine; a vaccine against *M. bovis*, Myco-B One-Dose™ (American Animal Health, Grand Prairie, Texas, USA), was developed in the United States for treating youngstock. Vets in the UK could apply for a special import license to use on farms, though its use was still limited. There was limited data available on the use of this vaccine with only one study reporting the effectiveness of the vaccine on a handful of dairy herds in Aberdeenshire in Scotland (Fowlie, 2021).

1.8.2 Hygiene practices during calf feeding

Biocontainment within a farm is essential to minimise the risk of disease transmission between groups of animals (Robertson, 2020).

Contamination of milk and colostrum can occur at any point from collection to feeding (Fecteau et al., 2002), therefore it is essential that the entire feeding regime of youngstock is conducted hygienically. Poor cleaning of the udder pre-milking can result in colostrum with a high bacterial load; therefore, it is essential to clean the udder prior to milking off colostrum for calves (Stewart et al., 2005) and limit the risk of *M. bovis* transmission from dam to calf.

Poor condition of the feeding equipment, i.e. tubes; teats; bottles and buckets can cause damage if they have any sharp edges or harbour harmful pathogens. It is recommended that feed buckets, teats and tubes are cleaned after every use to ensure there is no visible dirt, then left out to dry (Barry et al., 2019; Grothe and Thornsberry, 2022).

1.8.3 Pasteurisation of milk and colostrum

As well as ensuring that calf feeding equipment is cleaned and disinfected, cows' milk and colostrum can be treated to kill *M. bovis* and prevent its spread to calves. Butler et al. (2000) heat treated waste milk from cows receiving antibiotics or that were sick. *M. bovis* was killed after two minutes of treatment at 65°C, and one minute of heat treatment at 70°C inactivated the pathogen. Similarly, Stabel et al. (2004) reported that *M. bovis* was not recovered from

milk that was pasteurised at 71.7°C for five seconds. The same is true for pasteurising colostrum. No viable *M. bovis* was detected from *M. bovis*-inoculated colostrum following 30 minutes of heat treatment at 60°C (Godden et al., 2006).

Although pasteurisation kills or significantly reduces most harmful pathogens to manageable levels, it does not make colostrum and milk with high SCCs fit for feeding to calves (AHDB Dairy, 2020). Therefore, milk and colostrum from mastitic cows should not be pasteurised and fed to calves as the quality cannot be improved through heat treatment.

1.9 Prevalence studies

Many *M. bovis* prevalence estimates have been published, though it's not always possible to compare findings due to different study designs, farm systems, the types of samples collected, diagnostic tests used, and the age of cattle sampled from. Prevalence estimations may differ between countries.

Various BTM prevalence estimates have been reported, based on the presence of active infection, detected by PCR. Most are relatively low, such as 0% in Canada (Bauman et al., 2018), 1.5% in Belgium (Passchyn et al., 2012), 0.9% in Australia (Morton et al., 2014) and 3.8% in Japan (Murai and Higuchi, 2019). In the USA, between 2019 and 2019, 79.7% of BTM samples submitted to the Quality Milk Production Services at Cornell University tested positive for *M. bovis* (Gioia et al., 2021). Detecting active *M. bovis* infection in BTM can be challenging due to the intermittent shedding patterns of *M. bovis* from infected cows. Consequently, these *M. bovis* prevalence estimates may be lower than the true prevalence. In terms of herd-level antibody prevalence, there were few published BTM estimates at the time of commencing this PhD. For instance, the estimated BTM prevalence in Belgium was 24.8% (Gille et al., 2018) and in Ireland the prevalence ranged between 0.42-53% (McCarthy et al., 2021). Also, out of 120 Estonian dairy herds, 20% were antibody positive in at least one BTM sample (Mõtus et al., 2021).

The reported prevalence estimates in youngstock vary, ranging from 0-100% (Hanzlicek et al., 2011; Pardon et al., 2011; Radaelli et al., 2008; Wiggins et al., 2007). It should be noted that most prevalence studies in youngstock to date

were done in beef calf rearing units, such as in the USA, which are not directly comparable to UK dairy herds. Although, the systems could be likened to dairy bull and some beef rearing systems in the UK, where youngstock from different sources are reared in one system. Furthermore, these studies vary by design, with differing numbers of samples, age groups sampled, types of samples (i.e. nasal swabs, blood, etc.), frequency of sampling, and diagnostic tests used. Consequently, it is important to conduct prevalence studies in different counties, as the findings in one study, and country, may differ greatly from another.

One country in which *M. bovis* has had a major impact in recent years is New Zealand, which was previously thought to be free from the pathogen. Following detection in 2017, a national eradication programme was initiated in late 2018 requiring the entire dairy industry to partake in BTM testing (Laven, 2019). At the time of writing this thesis, the country was still in the delimiting phase of the programme, which involves network and background surveillance (Ministry for Primary Industries, 2023).

There are currently no national prevalence estimates available for *M. bovis* in Scotland nor the UK, and there is limited data available about *M. bovis* cases in Scotland. Between 1995-2005, Lawes et al. (2008) carried out an epidemiological study of *M. bovis* in Britain and reported that 42.4% of herds in England and Wales were infected with *M. bovis*. Following a decrease from 2012 to 2013, the diagnosis of *M. bovis*-pneumonia in submissions in Scotland reported in the Great Britain Veterinary Diagnostic Network has been steadily increasing from 2013 to 2019, before decreasing again in 2020 (GB Veterinary Diagnostic Network, 2023) (Figure 1-1). There was a gradual increase in the proportion of pneumonia submissions associated with *M. bovis* to 16% in 2019, whereas the proportion of *M. bovis* mastitis submissions remained below 3%. The proportion of arthritis submissions in which *M. bovis* was diagnosed as the cause were particularly high compared to mastitis and pneumonia submissions, however this is because the total number of all arthritis submissions was very low.

The increase in *M. bovis* pneumonia submissions could imply that the prevalence of this disease is increasing throughout the country. However, a caveat with this data is that it comprises only of voluntary submissions to veterinary post-mortem

facilities throughout the country and therefore cannot be used to estimate the prevalence. Furthermore, it is not known if the number of *M. bovis* diagnoses came from samples/animals with multiple pathogen infections or only with *M. bovis*. The increase was likely due to increased testing and submissions relating to *M. bovis* resulting from an increased awareness of the disease among farmers and vets.

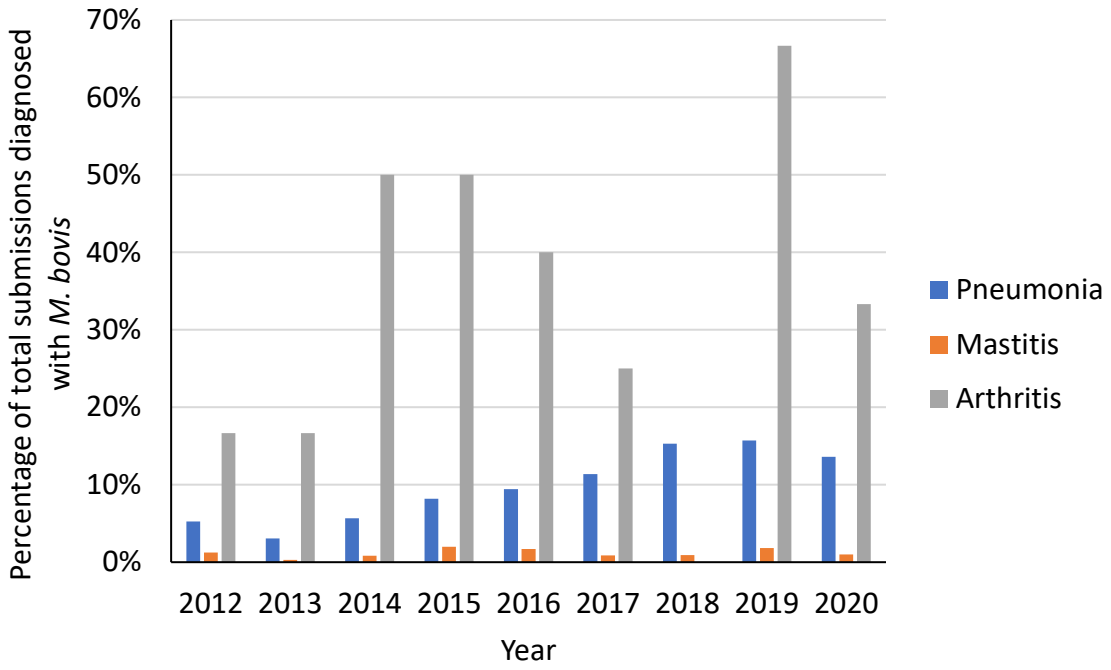


Figure 1-1: The proportion of total diagnoses of pneumonia, mastitis and arthritis in Scotland that were caused by *M. bovis* as reported in the Great Britain Veterinary Diagnostic Network between 2012 and 2020

1.10 Knowledge gaps

Although in recent years there have been many studies contributing towards our understanding of *M. bovis*, there are still knowledge gaps relating to the disease itself, prevention measures, and methods of control, many of which were summarised in a gap analysis by (Calcutt et al., 2018).

Prior to commencing the research that forms this thesis, the prevalence of *M. bovis* in Scottish dairy herds was unknown, and no previous research had been carried out on *M. bovis* in Scotland. At the point of commencing this PhD, *M. bovis* was a 'hot topic' in the Scottish dairy industry, being increasingly discussed at local farmers' meetings and in farming and veterinary publications.

Given the knowledge gaps and the industry's demands, establishing a foundation for *M. bovis* research in Scotland was essential.

Chapter 2 Methods

This chapter describes the general methods used in the BTM prevalence study (Chapter 4) and calf seroprevalence study (Chapter 6) of *M. bovis* in Scottish dairy herds.

Prior to commencing the study, ethical approval was obtained from the local ethics committee at the University of Glasgow School of Veterinary Medicine.

2.1 Source and target population

Dairy farms based in Scotland were the source and target populations for the study. Data referenced in this section was taken from the 2020 Scottish Dairy Herd Analysis unless stated otherwise (SDHA, 2020).

The main concentration of dairy herds in Scotland is towards the southwest of the country where the climate and topography can support the production of grass. Dumfries and Galloway, Ayrshire, and Lanarkshire are the three largest dairy producing regions (Table 2-1).

Table 2-1: The distribution of dairy cows and herds in Scotland by region as of 1st January 2020

Regions	No. herds by region	Percentage herds by region (%)	No. cows by region	Percentage cows by region (%)
Aberdeenshire, Angus & Moray	37	4	8,266	5
Ayrshire	220	25	35,763	20
Argyllshire	39	4	5,445	3
Highlands	4	0	370	0
Stirlingshire & Clackmannanshire	37	4	7,244	4
Perth & Kinross	7	1	1,664	1
Fife	20	2	3,658	2
Dumfries & Galloway	340	39	86,445	48
Orkney & Shetland	21	2	2,160	1
Dunbarton & Renfrew	31	4	4,400	3
Lanarkshire	94	11	15,897	9

Regions	No. herds by region	Percentage herds by region (%)	No. cows by region	Percentage cows by region (%)
Lothian	17	2	2,226	1
Scottish Borders	12	1	4,952	3
Total	879	100	178,490	100

Throughout Scotland, dairy production ranges from extensive grazing to intensively reared cows housed year-round (March et al., 2014). Most herds in the UK calve throughout the year, and a smaller proportion of herds calve in spring and/or autumn blocks (Giles et al., 2022). The choice of housing and management approaches appears to depend on the famers' length of time in farming and level of education (Borelli et al., 2023; Shortall and Lorenzo-Arribas, 2022).

2.2 Longitudinal bulk tank milk prevalence study

2.2.1 Study design and sample size calculation

A longitudinal BTM study was designed comprising four sampling points evenly distributed throughout one year.

Criteria for selecting the participating farms were as follows: dairy farms with cows that were located in Scotland.

The sample size formula was as follows:

$$Z = \frac{(Z2 \times P \times (1 - P))}{e2}$$

Where Z is the value from standard normal distribution corresponding to the desired confidence level, P is the expected true proportion, and e is the desired precision.

The sample size calculation indicated that a minimum of 88 farms was required to estimate the prevalence in a population size of 880, assuming a perfect diagnostic test, an expected prevalence of 50%, a desired level of confidence of 95% and precision of 10% (Sergeant, 2018a).

2.2.2 Recruitment of participants

Farms were recruited on an 'opt in' basis, therefore emphasis was put on increasing awareness of the study on as many platforms as possible.

The BTM prevalence study was launched in February 2020 via a press release on Scotland's Rural College (SRUC) website and social media platforms Facebook and Twitter. An email was sent to veterinary practices via SRUC Veterinary Services 'On the Hoof' monthly newsletter email. Veterinary practices that specialised in large animals, SAC Consultancy offices and SRUC Veterinary Services were contacted to encourage their clients to register for the study.

To minimise the potential for selection bias, flyers were sent to every dairy farm in Scotland inviting farms to participate between 3rd March 2020 and 24th March 2020 via the Scottish Dairy Hub mailshot (as shown in Appendix 1). Flyers highlighted the purpose of the study and included details on how to register interest.

As a result of the 2020 COVID-19 pandemic, the field study was temporarily suspended. Farmers who were already registered for the study were informed of this. On 24th August 2020, the study was relaunched with a second press release via SRUC and an article in the Scottish Dairy Hub and the Scottish Farmer. The deadline for farmers to express their interest in the study was initially set as the 30th of November 2020, however this was extended to December 2020 as recruitment was sporadic in the relaunch. An email was sent out to various dairy companies and vet practices in October 2020 with details of the study and routes for farmers to register. During the second round of recruitment between August and December 2020, a number of participating farms were registered for the study by their vet practice or dairy company.

2.2.3 Components of sampling kits

Upon receiving an expression of interest from the farmers with their contact details, the first sampling kit was posted out to the farmer. The first kits were posted in August 2020, and subsequently, kits were posted out to new recruits when the individual farm's details were received.

In each sampling kit, two tubes for sampling the BTM were enclosed, both containing a Bromopol preservative tablet. A zip lock bag was included to

prevent milk leakage during transport. Self-seal envelopes were provided with pre-paid second class return labels.

The first sampling kit contained a cover letter, participant information sheet, consent form, 3-page questionnaire and submission form (Appendix 2). The cover letter contained detailed information on the study including instructions on the sampling protocols and form completion, contact information of primary researchers, and concluded with guidance on returning the forms and samples. The participant information sheet covered the purpose of the study, emphasised how participants' data would be protected and used under the General Data Protection Regulation 2018, and noted that participants have no obligation to continue in the study if they did not wish to do so.

The subsequent three sampling kits contained a cover letter, submission form and appropriate BTM sampling components.

In the final sampling kit, there was a close-ended question provided on an additional piece of paper stapled to the front of the submission form asking if participants would be willing to take part in the next phase of the study.

2.2.4 Minimising participant dropout

To maintain engagement and reduce drop-out of participants, after each BTM sample was tested, the results were sent to the farmer in the post and emailed to their registered vet practice. Participants were sent reminders ahead of the subsequent sampling period via text or email. Subsequently, sampling kits were posted to participants ahead of their sampling period with instructions on when to aim to collect their samples. Additionally, *M. bovis* factsheets were included in the subsequent three sampling kits (Appendix 3).

2.2.5 Project forms

Construction of questionnaire

The purpose of the questionnaire was to identify potential risk factors associated with the presence of *M. bovis* in BTM. Themes explored in the questionnaire related to gaps in the general understanding of *M. bovis* as well as some previously identified risk factors; herd size and structure, cow housing, calving,

calf rearing and management including feeding and grouping, milk and colostrum management, and herd history of *M. bovis*.

The number of questions was restricted to three pages to minimise the time taken to complete and to enhance participation.

The questionnaire contained 16 closed questions, four of which were two-part questions, amounting to a total of 20 questions. Two questions contained text boxes for participants to input free written text. The remaining 18 questions were provided with boxes for participants to place a tick or cross to indicate their responses. Five questions had the option of an empty "other" field and space to write an additional response that the participant did not feel fitted into the pre-supplied options. The final question contained a space to write additional information if the participant selected "Yes". Six questions were multiple choice, and the remaining questions were restricted to a single response.

The questionnaire was piloted on a small number of farmers and vets prior to data collection. Amendments were made before sending the final questionnaire to the BTM study participants.

Sample submission form

The submission form comprised of six questions to capture information on the BTM samples that were collected, Appendix 2. Five close-ended questions were provided with space for participants to write their response; the date of sample collection, nearest SCC to the time of sample collection, the number of cows contributing to the bulk tank sample, the number of cows not contributing to the tank due to illness or treatment, and the number of dry cows at the time of sampling. One open-ended question was provided at the end of the submission form asking for participants if there were any changes since the previous sampling point and allowing participants to include any other information that they deemed important.

2.2.6 Sampling protocol

Farmers were provided with guidance on the sampling procedures. In short, participants were asked to ensure that milk was agitated prior to sample collection and to collect the samples in another vessel as the tubes provided

contained a preservative tablet. Participants were asked to fill the tubes almost to the top from their BTM. They were provided with a zip-lock bag to prevent the sample tubes leaking during transport. The samples were then sent by the participants to SRUC (Scotland's Rural College) Disease Surveillance Centre in Dumfries using a pre-paid postage envelope.

2.2.7 Sample processing and testing

Sample processing

Samples were booked into the Laboratory Information Management System and labelled upon arrival at the SRUC (Scotland's Rural College) Disease Surveillance Centre in Dumfries. They were frozen at -20°C prior to being sent to the SRUC Veterinary Services Veterinary and Analytical Laboratory, Edinburgh for testing in batches.

Sample testing

All of the BTM samples from all farms were subject to molecular testing using PCR for the presence of *M. bovis* DNA and serological testing for the presence of anti-*M. bovis* antibodies. The enzyme-linked immunosorbent assay (ELISA) test gave an indication as to whether there had been recent exposure to *M. bovis* whereas the PCR results signified an active infection at the time of sampling.

PCR:

The PCR for *M. bovis* was carried out in the commercial lab according to their own protocol using the Applied Biosystems VetMAX *M. bovis* kit. A brief summary of the protocol is as follows.

The DNA extraction method uses the MagMAX Express 96 Particle Processor with the MagMAX 96 Viral RNA Isolation Kit (Applied Biosystems/ Life Technologies).

The *M. bovis* real time PCR assay utilises the LSI VetMAX™ *Mycoplasma bovis* kit (Life Technologies/Thermo Fisher Scientific), which amplifies a target DNA sequence specific for *M. bovis* in the polC gene. The kit also amplifies an endogenous internal positive control (IPC) which should be present in every reaction containing a clinical sample. IPC amplification confirms successful nucleic acid extraction and the absence of PCR inhibitors, thus reducing the possibility of false negative results. The kit is deemed suitable by the

manufacturer for use with nucleic acid extracted from bovine milk, synovial liquids, trachea-bronchial liquids, organs and colonies.

The kit comprises of a ready-to-use mix which includes two sets of primers; one set specific for the *M. bovis* target sequence and the other set specific for the endogenous IPC, as well as a TaqMan® probe labelled with the fluorophore FAM™ NFQ specific for *M. bovis* and a second TaqMan® probe labelled with the fluorophore VIC®-NFQ specific for the endogenous IPC. The *M. bovis* real time PCR assay is run on an AB 7500 Real Time PCR System with an annealing temperature of 60°C and 45 amplification cycles.

The manufacturer has determined the limit of detection for the real time PCR assay with the LSI VetMAX™ *Mycoplasma bovis* kit to be 10 copies of nucleic acid per PCR. The measurement format for result interpretation is presence or absence of *M. bovis*. Samples are considered positive for *M. bovis* if the cycle threshold (CT) value is ≤ 37 and negative if the CT value is undetermined (no amplification). Samples with CT values > 37 but ≤ 45 are considered inconclusive and are initially retested.

ELISA:

BTM samples were also analysed with the ID Screen® *Mycoplasma bovis* indirect ELISA kit according to the manufacturer's instructions (IDvet, Grabels, France). All reagents, including positive and negative controls, were included in the kit provided by the manufacturer. The samples were diluted 1:40 in dilution buffer in the pre-coated plates. Positive and negative controls were added in duplicate to each plate. After incubation for 45 min at room temperature each well was washed three times with wash solution prior to addition of 100 µL anti-bovine horseradish peroxidase (HRP) conjugate. After being incubated for 30 minutes at RT, the plates were again washed three times before 100 µL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well. The plates were incubated 15 min in the dark at RT before the reaction was stopped by adding 100 µL stop solution. The optical density (OD) was measured at 450 nm. The test was considered valid if the mean value of the positive control was greater than 0.350, and the ratio between the mean positive control and the mean negative control was greater than three. For each serum sample the sample-to-positive percentage (S/P %) was calculated using the formula:

$$S/_P\,\% = \frac{OD_{sample} - OD_{mean\ negative\ control}}{OD_{mean\ positive\ control} - OD_{mean\ negative\ control}} \times 100$$

The S/P % for each sample for each run was used to categorise the sample as positive or negative using the cut-off value provided by the manufacturer (positive if the S/P % ≥ 30%).

The manufacturers of the ELISA test quote sensitivity and specificity to be 95.7% and 100%, respectively, though the specificity is more likely 98% (C. Mason, Personal Communication).

ELISA test results were expressed as both a qualitative (positive/negative) and a quantitative (optical density) value.

2.2.8 Data processing and cleaning

Unique identification codes were assigned to each participant on receiving the first sampling kit. All documents received, including each page of the questionnaire were labelled with the unique ID code. All questionnaire and submission forms received were considered useable if at least one question had been answered.

All forms were scanned and saved as PDFs. A Microsoft Excel file was created containing a list of all participating farms and the assigned unique ID code.

Questionnaire and submission form responses were entered into a Microsoft Access file upon arrival and then transferred to Microsoft Excel at the end of data collection.

Once the data was cleaned and checked, it was imported in RStudio (Posit team, 2022) for analysis.

2.2.9 Statistical analysis

Case definitions

Depending on the question, case definitions were based on the results of the two diagnostic tests:

1. Positive for the presence of *M. bovis* by PCR

2. Antibody positive by ELISA using manufacturers cut-off of 30%

Risk factor analysis

All data analyses were performed in RStudio. Specific packages used are given in *italics*. Various visualisations of the data were created using *vcd*, *sjPlot*, and *ggplot2*. BTM results were firstly treated as a categorical variable, either positive or negative, based on the optical density cut-off value of 30% for the *M. bovis* indirect ELISA test, then as a continuous variable. Normality of discrete/continuous data in the questionnaire and submission form, and the BTM optical densities was tested using the Shapiro-Wilk test in RStudio.

Following descriptive analysis of the questionnaire and submission form data, multiple choice question responses were recategorized to reduce the number of individual factor categories for the univariable analysis. Re-categorisation of categorical question responses was based on biologically plausible explanations, i.e. grouping dates of sample collection to months or seasons, and categorising all forms of calf feeding as either individual or group feeding.

The association between the binary BTM results and independent variables taken from the questionnaire were evaluated using Chi-square test for association. Statistical significance was set at a 95% confidence level with a p-value of ≤0.05. Mosaic plots were created using *ggmosaic*, an extension of *ggplot2*.

After initial Chi-square testing of categorical variables in the questionnaire, further regrouping of individual factor categories was necessary, due to small numbers of expected values in some cases.

Variables were then analysed using binary logistic regression analysis. The odds ratio (OR) for each category was calculated and presented with the 95% confidence interval (CI) and associated P value for the test. The reference level chosen for each variable was the one that made most biological sense.

To check for multicollinearity prior to conducting multivariable analysis, the variance inflation factor (VIF) was calculated between variables in each model. A correlation matrix was also created using *corrplot* to assess collinearity between all explanatory variables in the multivariable models. Where variables were strongly correlated with each other, the variable that was of less value of

the two was removed from the models. BTM was then analysed as a continuous variable using linear regression modelling.

Variables were then analysed at the multivariable level, initially including explanatory variables from the univariable analyses with a P<0.2. Multilevel generalised linear models were fit using the R package *lme4*.

A forward stepwise selection process was used first to retain covariates in the multivariable logistic regression model. Explanatory variables were retained in the model if they significantly improved the model fit. An automated stepwise selection process was also used for comparison.

The selection of the final model was based on the combination of variables that explained most of the data with the least number of covariates (i.e. the most parsimonious model). This was verified by computing the AICc. The AICc is a modification of the AIC for small sample sizes using the package *AICcmodavg*. The model with the lowest AICc was accepted as providing the best fit to the data.

2.3 Cross-sectional calf seroprevalence study

A cross-sectional study design was used to investigate the seroprevalence of *M. bovis* in youngstock, identify potential risk factors associated with the presence of *M. bovis* and examine the association between the BTM prevalence and seroprevalence of *M. bovis* in calves.

2.3.1 Study design and sample size calculation

A cross-sectional calf seroprevalence study was designed to sample from a subset of the herds that participated in the longitudinal BTM prevalence study (Section 2.2). A sample size of seven calves was required where if no seropositive calves are found, the probability that the population is seropositive at a prevalence of 50% or more is 1% (Cannon and Roe, 1982).

The study was carried out under the Veterinary Surgeons Act 1966 and was used as part of herd health planning. No farmers were forced to participate in the study, and this was an opportunity for farmers to receive free testing.

2.3.2 Selection of participating farms

The eligibility criteria for the calf seroprevalence study required farms to be willing to participate in the study, have completed three or four sampling periods in the BTM study, and not use a vaccine against *M. bovis* within the farm.

Farms that submitted three or four samples in the BTM study were categorised as either consistently positive, consistently negative, or transitional (both positive and negative results).

Due to funding limitations and to enable more detailed investigation of farms, the desired number of farms for the calf seroprevalence study was 60, with an even split of 20 herds from each of the three categories. The sample size for this phase of the study was based on maximising the diversity of farms sampled, according to the results of phase 1, within the limitations of resources available.

At the end of the BTM prevalence study, participants were asked if they were interested in taking part in the calf seroprevalence study using an interest slip and recruitment letters (Appendix 4). In total, 89 participants from the BTM study expressed their interest in the calf seroprevalence study, eight of which used a vaccine against *M. bovis* in their herd. This resulted in a total of 81 farms that could be approached to participate in the study.

Forty-eight farms were in the consistently positive category, 14 were consistently negative, and 19 transitional. To ensure an even distribution of selection from the consistently positive herds, the mean optical density was calculated for each herd. The herds were then listed from lowest to highest based on this value. Two consistently positive groups were created, “low positive” and “high positive” based on the mean optical density value of all consistently positive herds (i.e. all BTM samples had an OD >30). Each group was then randomised in a list using the ‘rand()’ function in Microsoft Excel. The first 10 farms in each of the two groups were approached and asked to participate. As there were smaller numbers in both the consistently negative and transitional categories, all farms in these categories were asked to participate.

2.3.3 Contents of sampling kits

Sampling kits were initially posted out to veterinary practices who had clients recruited in the study.

The sampling kits contained blood tubes, needles and needle guards to sample 22 calves in total. This was to sample the 20 calves and provide two spare needles, blood tubes and needle guards in case they were needed. A tube for the BTM sample was included which contained a Bromopol preservative tablet. One large zip-lock bag and three small zip-lock bags were included to prevent spillage of samples onto documents.

A cover letter, participant information sheet, consent form, sampling protocol, sample submission form and questionnaire were enclosed in the sampling kits (Appendix 8).

2.3.4 Project forms

Construction of questionnaire

A short questionnaire was designed to conduct a more in-depth investigation of potential risk factors relating to calf rearing and management that are associated with the presence of *M. bovis* in calf sera. Questions were focused on calf housing, milk and colostrum feeding, and calving practices.

The questionnaire consisted of 18 questions, two of which were two-part and two were three-part, amounting to an aggregate of 23 individual questions. Fourteen questions were closed and nine open-ended. Of the 14 closed questions, three were multiple choice and 10 were single response questions. Four of the close-ended questions had the option for participants to elaborate on their response or to select ‘other’ and write a different response that was not present on the questionnaire.

Submission form

The submission form asked participants to input the date on which the samples were collected and some information on the calves that were sampled: identification of each calf in the form of ear tag numbers and which age category they were in. There was also space for participants to write which pen

the calves were housed in and the option to draw a rough schematic diagram of the housing setup to assess transmission of *M. bovis* on the back of the form.

2.3.5 Sampling protocols

Blood samples were collected from two cohorts of calves at each study farm: 4-8 months old and 10-14 months old. Sampling of calves was carried out by participants' vets.

Participants and vets were provided with a sampling protocol to ensure sampling was even across both age groups of calves. Briefly, participants were asked to randomly sample 20 homebred calves in total, 10 from each age group. Calves could be of any sex and breed, i.e. Holstein-Friesian or beef crosses. At the same time as sampling the calves, participants were asked to collect a BTM sample as described in Section 2.2.6. This was the fifth BTM sample collected by these farmers.

The samples, consent form, questionnaire and submission form were posted by the farmer using a pre-paid postage envelope provided by the researchers.

2.3.6 Sample processing and testing

Blood samples were labelled upon arrival at the SRUC Disease Surveillance Centre in Dumfries and refrigerated at 2-8°C until they were centrifuged using the Eppendorf 5810 at a rate of 4,000 rpm for five minutes. Sera was extracted from the samples and transferred into a new tube before being sent to SRUC Veterinary Services Veterinary and Analytical Laboratory, Edinburgh for serological testing. Sera and BTM samples were tested using the ID Screen® *Mycoplasma bovis* indirect ELISA kit as described in Section 2.2.7. Calf sera was considered positive if the S/P value was ≥ 60%.

Results were reported for each individual calf as a categorical (positive/negative) and a continuous (OD%) value. BTM test results were reported as positive (OD ≥ 30%) or negative (OD < 30%). A brief interpretation of the overall picture based on the calf and BTM results was also provided to each participant and their vet practice.

2.3.7 Data processing and cleaning

All forms were labelled with the unique ID of the farm which was carried over from the BTM prevalence study. Forms were scanned and saved as PDFs. Submission form and questionnaire responses were input into a Microsoft Access document which was later transferred to Microsoft Excel for data cleaning.

2.3.8 Case definition

Calf-level

The total number of seropositive and seronegative calves in the study were calculated where a positive calf was defined as a calf that tested antibody positive in the ELISA test and a negative calf tested seronegative according to the standard criteria.

Group-level

The total number of positive calves in each age group per farm, 4-8 months old and 10-14 months old, was calculated. An age group was defined as positive if there was at least one seropositive calf, and negative if there were no seropositive calves.

Herd-level

A herd was defined as positive if there was at least one seropositive calf. If all calves were seronegative then the herd was defined as negative.

To incorporate the differences within the age groups for each farm, herds were also categorised based on a combination of the results from each age group to produce four results:

- All positive
- All negative
- Positive 4-8 month-olds and negative 10-14 month-olds (‘PosNeg’)
- Negative 4-8 month-olds and positive 10-14 month-olds (‘NegPos’)

2.3.9 Statistical analysis of data

Risk factors associated with herd-level calf seroprevalence

All data analyses were carried out in RStudio (Posit team, 2022). Frequency tables were generated to calculate group sizes in the questionnaire responses. The responses were re-categorised to reduce the number of groups and create larger groups for analysis. Questions with very small groups that were unable to be grouped were not taken forward for the statistical analysis.

To identify potential risk factors associated with herd-level calf seroprevalence, questionnaire responses were compared to whether herds were positive or negative.

Chi-square testing was used to determine if there were any associations between the questionnaire responses and herd-level calf serostatus, with significance accepted at a confidence level of 95% and $p<0.05$.

Association between BTM and calf seroprevalence

The mean calf OD% was calculated for each farm, and the mean BTM OD% was calculated for each farm based on their four samples from the first study and then a second mean was calculated based on their four samples plus the fifth BTM sample collected in the calf study. To determine if there was a correlation between the BTM OD% and herd-level calf OD%, Kendall's rank correlation was performed on the data in RStudio.

Chapter 3 Descriptive analysis of study responses: recruitment, participation, and questionnaires

This chapter describes the results of two questionnaires conducted on dairy farms in Scotland. The first questionnaire was part of a longitudinal BTM prevalence study of *M. bovis* in dairy herds and the second study was a cross-sectional calf seroprevalence study.

3.1 Introduction

3.1.1 Changes to Scottish and UK dairy farming

Since 1974, the total number of cattle in Scotland has been on a steady decline from 2.68 million to 1.72 million cattle, followed by a slight increase in 2021 (Scottish Government, 2021). Dairy cow numbers have been gradually increasing from 164,000 cows in 2011 to 174,000 in 2021 (Uberoi, 2021), with the most popular breed being the Holstein-Friesian (HF). The southwest of Scotland, which contains the largest proportion of dairy herds in the country, is a very stable milk producing region, thanks to the optimal climate and grass growth (C. Mason Personal Communication). The increase in dairy cow numbers in Scotland was mirrored in the UK as a whole, with an increase from 1.80 million dairy cows in 2011 to 1.85 million in 2021 (Uberoi, 2021). National milk production has remained relatively stable, which is attributed to an increase in the efficiency of dairy cows (Oltenacu and Broom, 2010).

The structure of the Scottish dairy industry relating to calf rearing, milk production and breeding have evolved in recent years due to changes to UK and Scotland-specific policies, national legislation, and consumer preferences.

Dairy bull calves had low marketability in the UK and were thus considered a by-product of the dairy industry (Kirkland et al., 2006). Consequently, these calves were often euthanised to remove them from the herd. Understandably, this practice was not favoured by the consumer nor by many within the dairy industry itself. Recent changes to UK milk buyer policies and increased public concerns for the welfare of calves has driven more farmers towards rearing these calves themselves or selling them to rearing units (Rutherford et al.,

2021). The GB Dairy Calf Strategy 2020-2023 outlines the industry changes required to eliminate the euthanasia of calves by 2023 (AHDB, 2020).

Historically, exportation to the European Union (EU) for veal was an alternative market for UK dairy bull calves and reduced the need for euthanasia (Rutherford et al., 2021). Following the bovine spongiform encephalopathy (BSE) epidemic in the UK, the EU imposed a ban on the export of beef from the UK from 1996 to 2006 (Schreuder et al., 1997). In recent years, concerns have been raised regarding the welfare of animals during export, which has once again resulted in the live exports of calves being banned from the UK.

There is not a large market for veal in the UK, though with the development and commercialisation of sexed semen, paired with the advancements in genomics, dairy farmers can select for the best heifer calves and thus reduce the number of bull calves born (De Vries et al., 2008; Pryce and Hayes, 2012). Furthermore, after one or two cycles of AI, many farmers put their cows to a beef bull to produce dairy beef crosses that are of higher value. The dairy beef industry has grown so much that 60% of beef produced in the UK is in fact from dairy farms (C. Mason, Personal Communication).

Dairy bull-beef calves may be retained and reared on farm or sent to a calf rearing facility. One of the major issues with these rearing facilities is the risk of disease, including *M. bovis*. Some units will source calves from as many as 40-50 farms, which will all have different levels of immunity and exposure to pathogens. Depending on the set-up of the unit, calves of varying ages may be grouped together, potentially exposing younger, naïve calves to older calves with developed immune systems (Nordlund and Halbach, 2019). This heightened risk of disease is still much more favourable than euthanising male calves, however, it has led to the increased use of antimicrobials to protect calves upon entry to these rearing units.

Antimicrobial resistance (AMR) is another aspect of dairy farming that is constantly under the spotlight. Previously, it was common for farmers to ‘blanket’ treat all youngstock with antibiotics as a preventive measure (also known as prophylaxis) rather than only treating sick symptomatic individuals (Dumas et al., 2016). Metaphylaxis was also a common approach, treating all animals just prior to an expected outbreak. The overuse of antimicrobials in

both human and animal medicine has resulted in the existence of various bacterial strains that are challenging to treat due to being resistant against multiple antimicrobial classes (Amann et al., 2019). Currently, the biggest use of antimicrobials is in beef calf rearing units to prevent the introduction and spread of pathogens in the bovine respiratory disease complex, of which *M. bovis* is a part (Pratelli et al., 2021).

Unlike the USA where in most rearing units, calves are prescribed antimicrobials upon arrival to prevent respiratory disease, in the UK this is much less common (Machado and Ballou, 2022). In UK calf rearing units, treatment is generally targeted toward calves showing signs of respiratory disease instead of blanket treatment of all calves (RUMA, 2024). Any animal that receives antibiotics must not enter the food chain, nor must any produce from that animal (i.e. milk), until the withdrawal period has passed. Instead of discarding waste milk from a cow treated with antibiotics, this milk was fed to youngstock (Aust et al., 2013), which contributes to the rise of antimicrobial resistance (Foutz et al., 2018; Maynou et al., 2017). This practice is against the Red Tractor standards therefore should not occur; however, unfortunately waste milk is still fed to calves in a number of herds. The dairy industry as a whole is striving towards reducing prophylaxis and metaphylaxis and to instead target antimicrobial use to treat individual animals infected with disease.

The structure of the UK dairy industry is always evolving to meet consumer demands and changes in legislation. These changes are possible due to technological advancements and the industry's proactive approach and adaptability.

3.1.2 Farm surveys

Questionnaires are a commonly used method of collecting information on dairy farm management practices and their relationship with disease and production (Scholl et al., 1992). Rather than directly observing management practices on farm which is time consuming and not practical when studying a large number of farms, questionnaires are a more time-efficient alternative (Scholl et al., 1992). Dairy farmers are notoriously busy people, therefore short questionnaires that can be answered in around ten minutes are more convenient and more likely to

be completed by farmers (Edwards et al., 2002). Recent studies have provided important insights into UK dairy farmer practices relating to calving patterns (Giles et al., 2022), dry cow management (Fujiwara et al., 2018), and calf husbandry (Mahendran et al., 2022). National legislation policies, veterinary advice, and milk buyers also stipulate what farmers should be doing, though due to a lack of studies carried out only on Scottish dairy farms, it is not known what farming practices occur. Information on popular management practices in Scottish dairy farms is key to measuring changes in management practices and to evaluate the effect of on-farm interventions and new technologies on performance.

3.1.3 Chapter aim

The aim of this chapter was to describe the common structures and management practices of dairy farms in Scotland by reporting questionnaire results from the BTM study (Chapters 4 and 5) and the calf study (Chapter 6).

3.2 Methods

Two consecutive studies were conducted on *Mycoplasma bovis* in Scottish dairy herds. The first study was a BTM study that required farmers to submit four bulk milk samples three months apart that were tested for the presence of *M. bovis* and antibodies to *M. bovis*. Farmers were sent a short questionnaire on general herd management practices relating to the lactating herd and youngstock.

The second study was a calf seroprevalence study (calf study) that involved blood sampling of 20 calves per farm as well as an additional BTM sample all of which were tested for the presence of antibodies to *M. bovis*. The sampling was accompanied by a questionnaire focusing on youngstock management and housing.

Further details on the recruitment, study design, and questionnaire design for both studies are described in Chapter 2.

Chi-square tests were performed on a small number of questionnaire and sample submission form responses to determine if there were any associations between

them. Fisher's exact tests were performed when the group sizes were less than five. This analysis was done in RStudio (Posit team, 2022).

3.3 Results – Bulk milk prevalence study

3.3.1 Participant demographics

Timeline of recruiting dairy farms

The timeline of promotional activities and the cumulative number of farms recruited at each timepoint is shown in Figure 3-1. Details of the promotional activities are described in Chapter 2. Just over half of the total participants (n=92) were enrolled during the first round of recruitment before the COVID-19 pandemic and the remaining farms (n=89) were recruited after the project was relaunched.

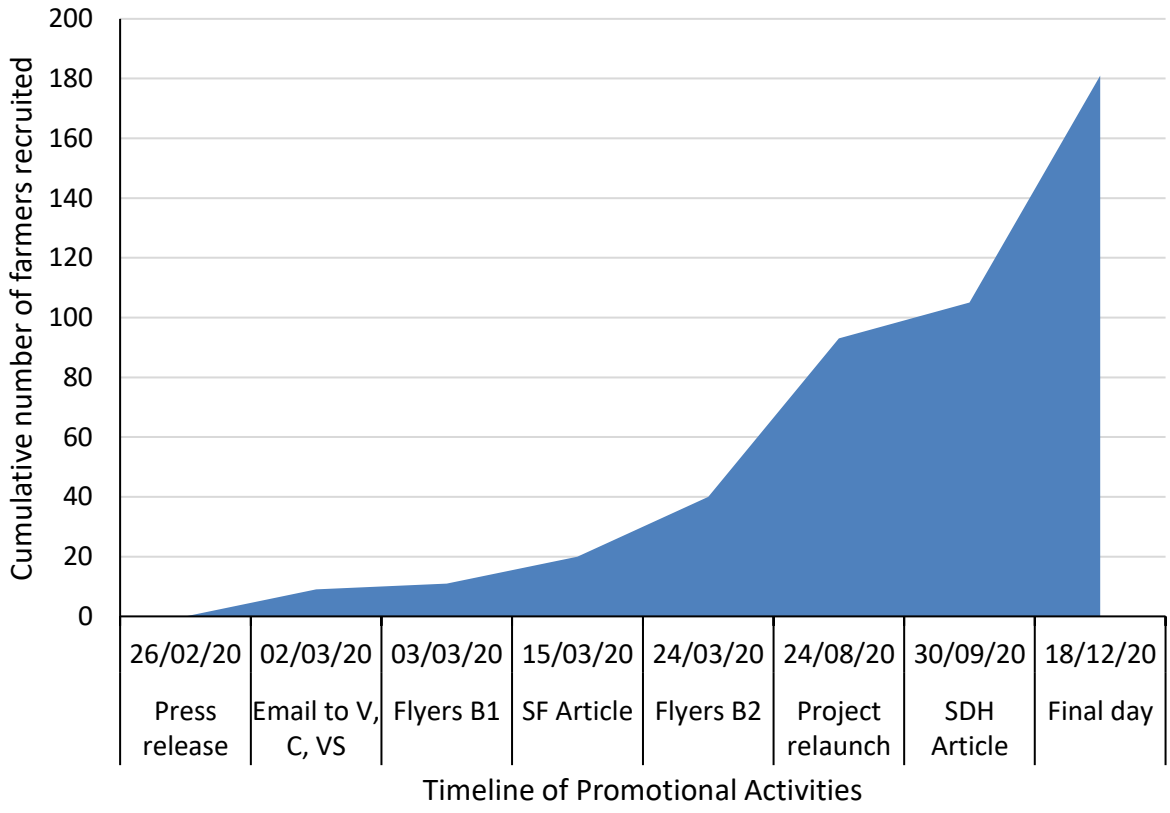


Figure 3-1: Timeline of recruitment activities for the BTM prevalence study and the cumulative number of herds enrolled at each activity. V = vets, C = SAC Consulting, VS = SRUC Vet Services, B1/B2 = batch 1/2, SDH = Scottish Dairy Hub.

A Chi-square test was performed to assess whether the farmers' awareness of *M. bovis* prior to participating in the study could have influenced the time when

farmers were recruited (pre- or post- COVID-19 lockdown). There was no association between awareness and the recruitment period (p=0.52) (Table 3-1).

Table 3-1: Association between the farmers' awareness prior to participating in the study and whether they were recruited before or after the 2020 COVID-19 lockdown.

Awareness	Recruitment (%)		Total
	Before lockdown	After lockdown	
Not aware	15 (8)	18 (10)	33
Aware	76 (42)	71 (39)	147
Total	91	89	180

Methods of enrolment

There were a variety of routes by which farmers were able to enrol on the BTM study, by directly contacting the researchers (post, phone, and email), or indirectly via their registered vet practice or dairy company (Table 3-2).

The most common routes of enrolment were by email and post. A small percentage of participants were enrolled via encouragement from a dairy company they work with.

Table 3-2: The number of farms enrolled by each recruitment method

Route of enrolment	N (%)
Email	62 (34)
Post	61 (34)
Vet	27 (15)
Phone	21 (12)
Company	8 (4)
Other	2 (1)
Total	181

Dropout prior to commencement of the BTM study

Eleven farms enrolled on the BTM study and dropped out prior to sending in their first samples. Seven of these 11 expressed interest in the first round of recruitment prior to the COVID-19 pandemic. Two of these farms were unable to participate when sampling finally began as the herds had been sold. The other

four herds that expressed interest in the second round of recruitment were not contactable after they expressed their interest.

Samples from three unknown farms were received with no farm name, it was suspected that these samples came from three of the four farms from the second recruitment. Despite contacting every farmer who expressed interest, the researchers were unable to match these samples to a farm and consequently they were not included in the BTM study.

Geographical distribution of dairy farms

One hundred and eighty-one dairy farms participated in the BTM prevalence study which was approximately a fifth (21%) of the total dairy farm population in Scotland at that time (SDHA, 2021). Scottish regions were grouped together to maintain confidentiality of participating farms due to the small numbers of farms in some regions (Table 3-3).

Table 3-3: Number and distribution (%) of dairy farms (total) and study dairy farms in Scotland by region

Region	Number of study farms in each region	Total number of farms in each region	Percentage of farms sampled from the total number of dairy farms within each region (%)
Aberdeenshire, Angus & Moray	6	37	16
Ayrshire	40	220	18
Argyllshire	7	39	18
Scottish Highlands	0	4	0
Stirling & Clackmannanshire	8	37	22
Perth & Kinross	5	7	71
Fife	4	20	20
Dumfries & Galloway	81	340	24
Highlands, Orkney & Shetland	3	25	12
Dunbartonshire & Renfrewshire	4	31	13
Lanarkshire	16	94	17
Lothian	4	17	24

Region	Number of study farms in each region	Total number of farms in each region	Percentage of farms sampled from the total number of dairy farms within each region (%)
Scottish Borders	3	12	25
Total	181	879	21

The highest proportion of participating dairy farms was in Dumfries and Galloway (45%), followed by Ayrshire (22%) and Lanarkshire (9%). According to the Scottish Dairy Herd Analysis, the three largest dairy regions in Scotland are Dumfries and Galloway, Ayrshire and Lanarkshire with 39%, 25% and 11% farms, respectively (SDHA, 2021). Every region was represented proportionally, though there was some apparent overrepresentation, e.g. Perth and Kinross and Dumfries and Galloway. Underrepresentation occurred in Ayrshire, Dunbartonshire and Renfrewshire, and Lanarkshire.

3.3.2 Sample data

Number of samples submitted per farm

Twenty-three farms in the BTM study (13%) submitted the first sample only, followed by 25 farms (14%) that only submitted the first two samples. Twenty-seven farms (15%) submitted three BTM samples, and 106 farms (59%) submitted all four samples.

Time between samples

The time between each sample period was calculated for farms that reported the date at which the sample was collected (Figure 3-2). Where this information was not provided, the dates that the samples were received at the Dumfries Disease Surveillance Centre were used. Between samples one and two, and three and four, the median number of days were 84 and 94, respectively. The median number of days between samples two and three was considerably higher (n=215).

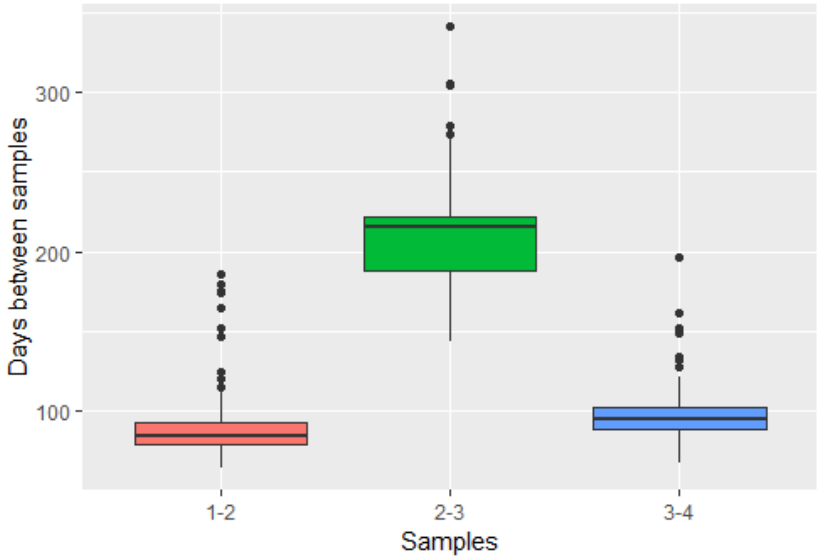


Figure 3-2: Number of days between sample points

The median number of days between the first and fourth sample was 308 (Figure 3-3).

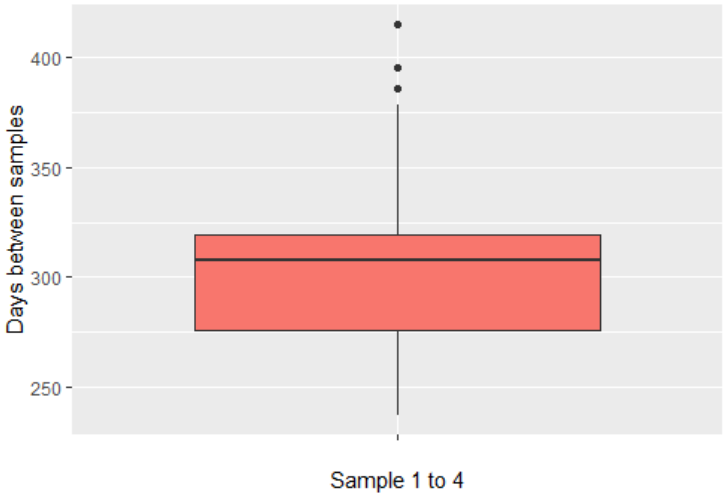


Figure 3-3: Number of days between sample one and sample four

Dropout throughout study

When testing for an association between the previous antibody test BTM result and whether herds continued to participate in the subsequent sampling point, there were no associations ($p>0.05$), as shown in Table 3-4. The BTM test results are presented in Chapters 4 and 5 of this thesis.

Table 3-4: Test for association between the results of the previous BTM sample and whether herds continued to the next sampling point

Variable	No	Yes	Odds Ratio	Confidence Interval	p
Outcome variable: continued to sample 2					
Negative BTM sample 1	3	41	1		
Positive BTM sample 1	24	113	2.89	0.81-15.78	0.093
Outcome variable: continued to sample 3					
Negative BTM sample 2	3	42	1		
Positive BTM sample 2	20	89	3.13	0.86-17.33	0.082
Outcome variable: continued to sample 4					
Negative BTM sample 3	4	19	1		
Positive BTM sample 3	24	88	1.29	0.38-5.72	0.784

3.3.3 Data collected at each sampling point

Somatic cell count

Participants were asked to provide their most recent BTM SCC at each sampling point (Figure 3-4). The SCC at the four sampling points ranged from 14,000 to 442,000. The mean SCCs were 148,062, 143,141, 141,852, and 155,087, for samples 1 to 4, respectively.

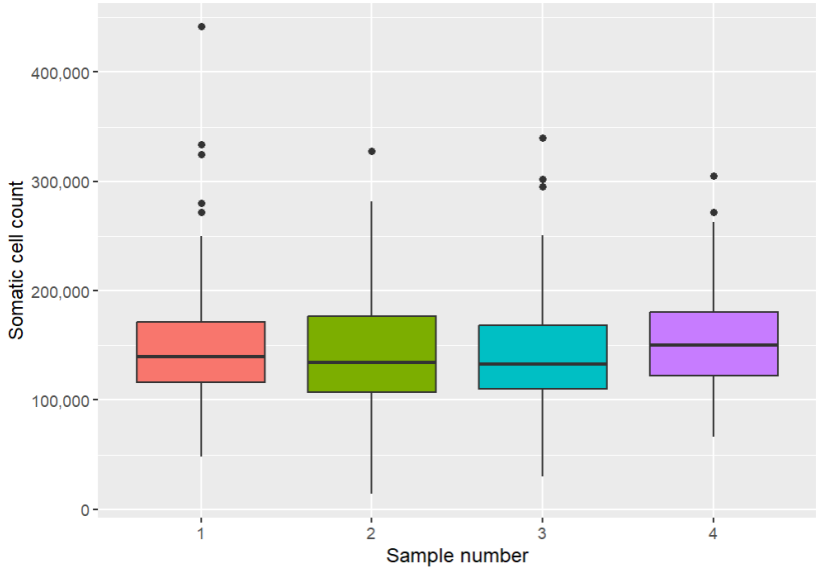


Figure 3-4: Distribution of somatic cell counts at each sampling point

A BTM SCC of 200,000 cells/ml or greater is considered to be high and often associated with milk quality and subclinical mastitis incidence (Bradley and Green, 2005). Therefore, the BTM study herds were categorised into those with a BTM SSC of <200,000 and >200,000 cells/ml based on their BTM results (Figure 3-5). At each of the four sampling points at least 84% of the participants had a SCC of <200,000.

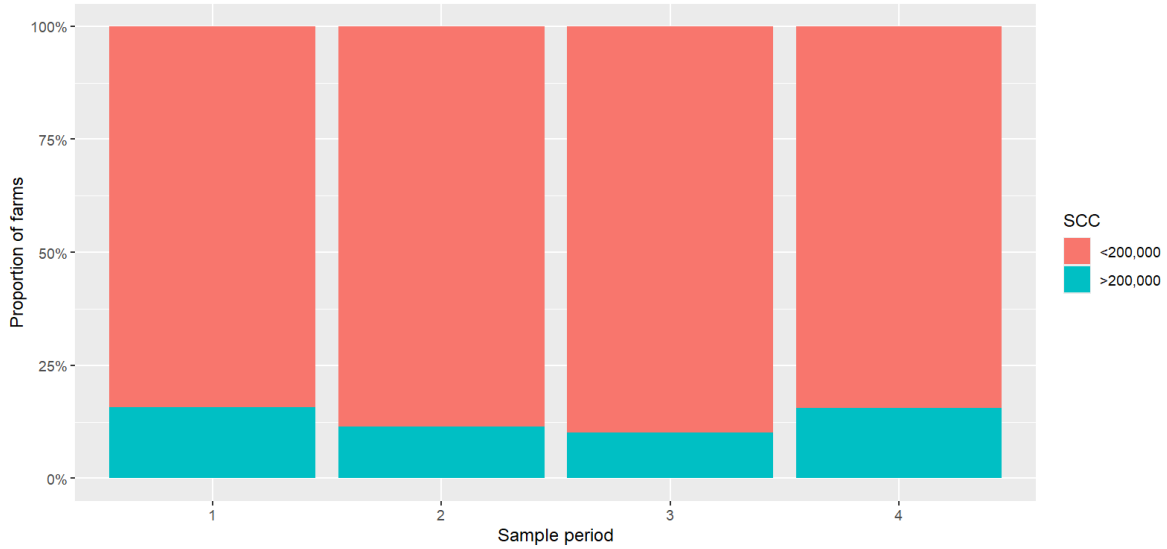


Figure 3-5: Proportion of farms with a somatic cell count of <200,000 and >200,000 at each sampling point

At each of the four sampling points a percentage of farmers did not report their most recent SCC. The percentage of missing data was 19% (35/181), 3% (5/154), 6% (8/135), 5% (5/108) from samples one to four respectively.

Cows numbers at the time of sampling

Three figures relating to the number of cows at the time of sampling were recorded by farmers at each sampling point: the number of cows contributing to the BTM, the number of dry cows, and the number of cows not contributing to the BTM due to illness or treatment.

As well as looking at the three sets of data individually, the sum of the number of cows contributing, cows not contributing due to illness or treatment, and the number of dry cows at each sampling point was calculated and compared to the number of cows recorded in the questionnaire as a form of internal validation (Table 3-5). Two outliers were removed from the sample 3 data; for one herd it was not clear what the number of cows that contributed was due to the way in which the farmer completed the sample submission form. The other herd was removed as the farmer separated the herd into two at this point therefore only some of the total herd contributed to the third BTM sample.

Table 3-5: Descriptive table of the difference in cow numbers between the questionnaire and submission form responses

Value	Sample 1	Sample 2	Sample 3	Sample 4
Minimum	0	0	0	0
Maximum	110	179	187	166
Range	110	179	187	166
Mean	8	15	19	19
Median	3	8	10	10
Quartile 1	1	4	3	5
Quartile 3	8	16	22	22
IQR	7	12	19	17

Proportion of cows contributing to the BTM

The percentage of cows in a herd that were contributing to the BTM at each sampling point is shown in Figure 3-6. The percentage contributing ranged between 43-100%, with the mean ranging between 84-87% and the median between 85-87% across the four sampling points.

As shown in Figure 3-6, there were a number of outliers with less than 70% of the lactating herd contributing to the BTM sample. In these herds, the reason for a lower proportion of the herd contributing to the BTM at the time of sampling was due to cows being dried off for calving.

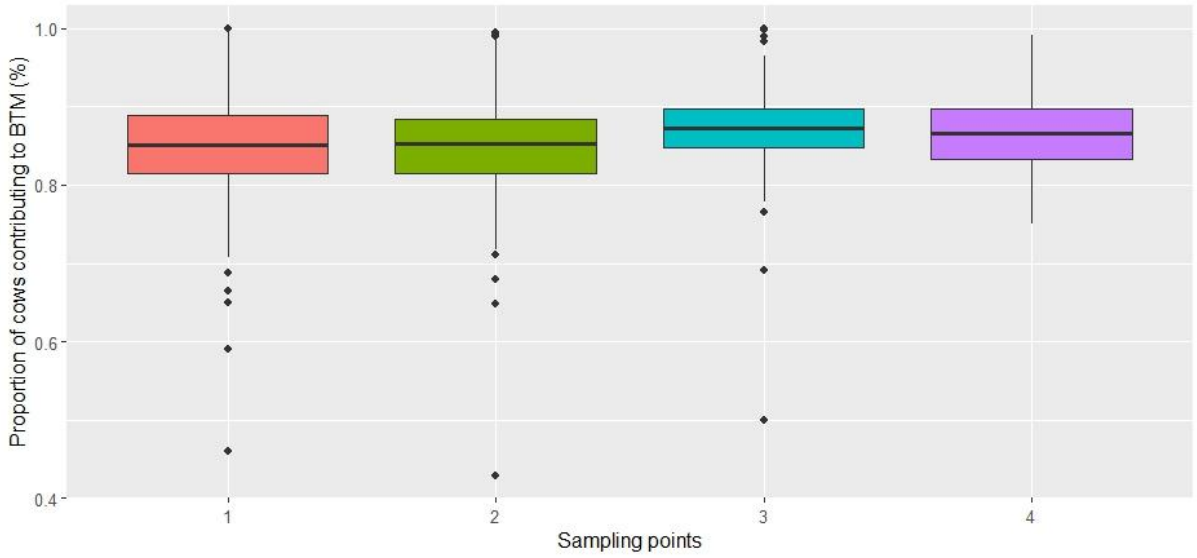


Figure 3-6: Distribution of the proportion of cows in a herd that were contributing to the BTM at each sampling point

Cows not contributing to the BTM due to illness or treatment

Farmers were asked how many cows did not contribute to the BTM at each sampling point due to illness or drug treatment. In some herds, there were no sick cows or cows receiving treatment at the time of sampling.

At each of the four sampling points the number of farmers that did not report the number of cows not contributing to the BTM was 14% (26/181), 1% (1/154), 0% (0/135), and 1% (1/108), respectively.

Number of dry cows

At each sampling point the minimum number of dry cows was 0. The maximum number of dry cows in herds at the time of sampling was 413, 400, 164, and 137, from samples one to four, respectively. The number of farmers that did not respond to this question at each sampling point was the same as the responses to the number of cows not contributing to the BTM.

3.3.4 Herd structure

Farmers were asked to state the number of cattle in the following categories: 0-12 month olds, 12-24 month-olds, cows, and bulls. Eight participants did not answer this question. The number of both the 0-12 and 12-24 month-olds ranged from 0 to 700, with a median group size of 106 and 90, respectively (Figure 3-7). The median number of cows per herd was 200, ranging from 57 to 1,168.

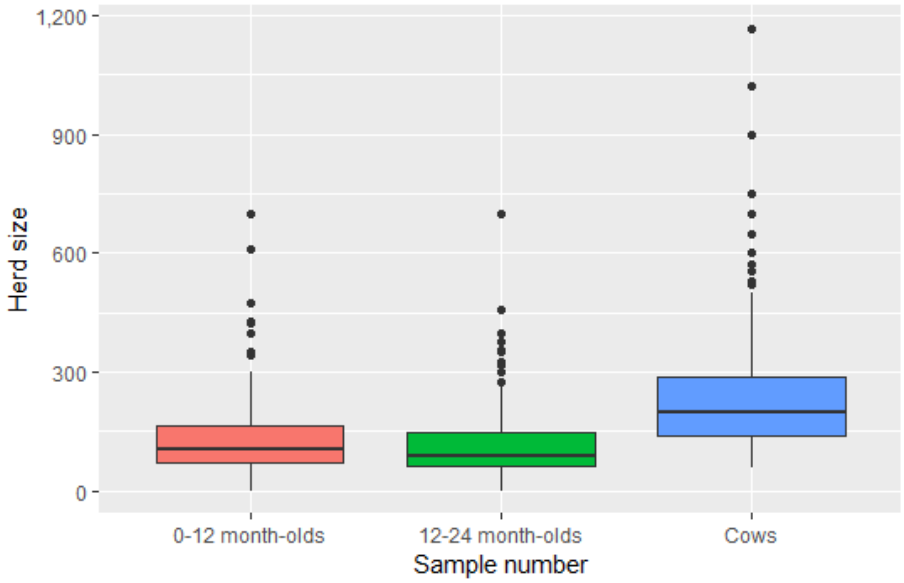


Figure 3-7: Distribution of the number of 0-12 month-olds, 12-24 month-olds, and cows

The number of 0-12 and 12-24 month-olds were then categorised into <100 and > 100 (Table 3-6). For both age categories there was almost an even split of herds with < 100 and > 100. The number of cows within study herds was categorised into < 200 and > 200. Just over half of the BTM study herds had more than 200 cows.

The number of bulls within herds ranged from 0 to 50 with a median of 1. Herds were then categorised into those with at least one bull or those with no breeding bulls. The majority of herds did not have a breeding bull on the farm.

Table 3-6: Questionnaire responses: questions relating to herd structure

Question number	Question topic	Categorised responses	N* (%)
Q1a	Number of 0-12 month-olds	< 100	76/173 (44)
		> 100	97/173 (56)
Q1b		< 100	96/173 (55)

Question number	Question topic	Categorised responses	N* (%)
	Number of 12-24 month-olds	> 100	77/173 (45)
Q1c	Number of cows	< 200	81/173 (47)
		> 200	92/173 (53)
Q1d	Have a breeding bull on farm	At least one	38/173 (22)
		None	135/173 (78)
Q3a	In last 12 months, bought any 0-12 month-olds	Yes	18 (10)
		No	163 (90)
Q3b	In last 12 months, bought any 12-24 month-olds	Yes	105 (58)
		No	76 (42)
Q3c	In last 12 months, bought any cows	Yes	43 (24)
		No	138 (76)
Q3d	In last 12 months, bought any bulls	Yes	69 (38)
		No	112 (62)
Formulated from Q3 (a-d)	In last 12 months, bought any animals	Yes	105 (58)
		No	76 (42)
Q4	Changing herd size	Increasing	51 (28)
		Maintaining	127 (70)
		Decreasing	3 (2)
Q2a	Do you rear dairy bull/beef calves	Yes	159 (88)
		No	22 (12)

*Denominator is 181 unless stated otherwise

As a proxy for whether herds were open or closed, a variable was created stating whether participants had bought in at least one animal from either of the four categories in the last 12 months. If a farmer did not answer this question then it was assumed that they had not purchased any cattle. These participants had completed all other questions. Only 10% of farmers had purchased 0-12 month-olds in the last 12 months, whereas 58% had purchased 12-24 month-olds. One quarter of the farmers had purchased cows, and 38% bulls.

Most farmers reported that they rear dairy bull and beef calves and only 12% did not (Table 3-7). Seven farmers who selected ‘no’ to Q2a responded to Q2b, making 166 responses. Only 19% of farmers sell calves before weaning, the

majority sell after weaning, and one quarter rear calves until slaughter. The remaining participants reported a combination of the options.

Table 3-7: Responses to Q2b: length of time rearing dairy bull and beef calves

Sell before weaning	Sell after weaning	Rear until slaughter	Other	N (%)
	X			80 (48)
		X		42 (25)
X				32 (19)
	X	X		6 (4)
X		X		3 (2)
X	X			2 (1)
			X	1 (1)
			Total	166

When asked if they were changing the size of their herd, the majority of participants were maintaining the size of their herd, while 28% were increasing and only 2% were decreasing their herd size (Table 3-8). Chi-square testing determined that the number of cows was not associated with whether farmers were changing the size of their herd ($P>0.05$).

Table 3-8: Comparison of the number of cows and whether the herd size was changing

Number of cows	Changing herd size			
	Increasing	Maintaining	Decreasing	Total
< 200	24	54	3	81
> 200	24	68	0	92
Total	48	122	3	173

Almost three quarters of participants (n=72) who had at least one breeding bull on farm said that they had not purchased a breeding bull in the last 12 months (Table 3-9). Three participants indicated that although they did not have a breeding bull at present, they had purchased at least one bull in the last 12 months. The remaining 35 farms that did not have a breeding bull had also not purchased a bull in the last 12 months.

Table 3-9: Comparison of whether there was a bull on farm and if a bull had been purchased in the previous 12 months

Bought a bull	Had a bull on farm		
	Yes	No	Total
Yes	63 (36)	3 (2)	66
No	72 (42)	35 (20)	107
Total	135	38	173

If a farmer had bought at least one breeding bull in the last 12 months, they had also bought in cattle from one or more of the other age groups (Table 3-10).

Table 3-10: Comparison of whether a bull had been purchased and if cattle had been purchased in the other three groups (0-12mo, 12-24mo, cows)

Bought bull	Bought 0-12mo, 12-24mo, or cows		
	Yes	No	Total
Yes	69 (38)	0 (0)	69
No	36 (20)	76 (42)	112
Total	105	76	181

3.3.5 Calving management

Almost three quarters of the participants did not use a separate calving pen while only 31% did use a separate pen (Table 3-11).

Table 3-11: Questionnaire responses: questions relating to calving management

Question number	Question topic	Responses	N (%)
Q6	Use of separate calving pen	Yes	57 (31)
		No	124 (69)
Q7a	Calving period	Year-round	158 (87)
		Block calving	23 (13)

The majority of participants operate a year-round calving pattern and the remaining 13% have a block calving pattern (Table 3-12). Farmers who block calve were asked what seasons they calve in. Almost three quarters of farmers calve in spring and/or autumn.

Table 3-12: Responses to Q7b: block calving season

Spring	Summer	Autumn	Winter	N (%)
X		X		8 (35)
X				6 (26)
		X		3 (13)
X		X	X	3 (13)
X	X			1 (4)
	X			1 (4)
	X	X		1 (4)
			Total	23

3.3.6 Housing management

Farmers were asked how their lactating herd are housed (Q5). Over half of farmers reported that they house their lactating herd seasonally, i.e. housed in autumn and grazed in spring and summer (Table 3-13). Twelve percent house their herd semi-permanently, either grazing at night and housed in the day or high producing heifers are housed and low producing heifers grazed. Three farmers selected both semi-permanent and seasonal housing. Cows were housed permanently in 29% of participating herds, and one farmer reportedly has both permanent and seasonal housing of the lactating cows. Only 3% of farmers operate on a maximum grazing system similar to New Zealand systems i.e. grazing the herd for most of the year.

Table 3-13: Responses to Q5: housing of lactating cows

Permanent housing	Semi-permanent housing	Seasonal housing	Maximum grazing	N (%)
		X		99 (55)
X				52 (29)
	X			21 (12)
			X	5 (3)
	X	X		3 (2)
X		X		1 (1)
			Total	181

When asked about calf housing, only 6% of farmers housed calves individually or in pairs until weaning (Q8). Most farmers housed calves individually or in pairs and then mixed them in groups before weaning (Table 3-14). Twenty-nine percent of farmers only housed pre-weaned calves in groups. Eight percent of farmers reported a combination of housing approaches.

Table 3-14: Responses to Q8: housing of pre-weaned, milk-fed calves

Individual or pairs until weaning	Individual or pairs then mixed	Group housing	N (%)
	X		104 (58)
		X	52 (29)
X			11 (6)
	X	X	11 (6)
X	X		2 (1)
X		X	1 (1)
		Total	181

Most farmers mix groups of calves post-weaning whereas over one quarter of farmers do not mix groups (Q9). Two percent of farmers reported that they both mix and do not mix different groups of post-weaned calves, and 3% try not to mix groups (Table 3-15).

Table 3-15: Responses to Q9: housing of post-weaned calves

Housing of post-weaned calves	N (%)
No mixing	50 (28)
Try not to mix	6 (3)
No mixing & mixing	4 (2)
Mixing	121 (67)
Total	181

3.3.7 Feeding practices

Most farmers feed cows' colostrum to calves, and 17% reportedly feed a combination of cows' colostrum and powdered colostrum (Table 3-16). Artificial milk was the most common choice of milk fed to calves, while 10% reported

feeding only cows' milk. Twenty-eight percent of farmers feed a combination of the two.

Of those that feed artificial milk, 82% feed cows' colostrum, and 18% feed a combination of artificial and cows' colostrum.

Table 3-16: Questionnaire responses: questions relating to calf feeding practices

Question number	Question topic	Responses	N (%)
Q10a	Colostrum source	Cows'	151 (83)
		Artificial and cows'	30 (17)
Q11a	Milk source	Artificial	113 (62)
		Cows'	18 (10)
		Artificial and cows'	50 (28)
Q10b	Pasteurise colostrum	Yes	11 (6)
		No	170 (94)
Q11b	Pasteurise milk	Yes	7/122 (6)
		No	115/122 (94)
Q12	How are calves fed pre-weaning	Individually fed	39/180 (22)
		Group fed	92/180 (51)
		Both	49/180 (27)

*Denominator 181 unless stated otherwise

Eight percent of farmers pasteurise colostrum and/or milk prior to feeding calves. Of those farmers, seven pasteurise colostrum only, three pasteurise milk only, and four pasteurise both.

Just over half of farmers feed calves by a combination of both individual and group feeding. Twenty-seven percent of farmers reported that they group feed calves, and only 22% feed calves using individual buckets and/or teats. One farmer did not answer the question.

A further breakdown of the methods of feeding calves pre-weaning are shown in Table 3-17. Feeding calves with an individual bucket was the most common

method, followed by a combination of an individual bucket and teat and an automatic feeder, an automatic feeder only, and a group feeding teat.

Table 3-17: Responses to Q12: how pre-weaned calves are fed

Individual bucket	Individual bucket & teat	Group teat	Automatic feeder	Group trough	Suckled	N (%)
X						34 (19)
	X		X			28 (16)
			X			26 (14)
		X				24 (13)
X				X		14 (8)
	X					14 (8)
	X	X				7 (4)
X	X					4 (2)
	X	X	X			4 (2)
	X			X		4 (2)
		X	X			4 (2)
X	X		X			3 (2)
X		X				3 (2)
X			X			3 (2)
X			X	X		2 (1)
				X		3 (2)
X	X	X				1 (0.6)
X	X			X		1 (0.6)
X					X	1 (0.6)
					Total	180

Due to a variation in responses, with farmers stating that they clean and/or disinfect feeding equipment, the frequency of cleaning was used for categorising responses. The frequency at which farmers reportedly clean feeding equipment varied (Table 3-18).

Table 3-18: Responses to Q13: frequency of cleaning calf feeding equipment

Housing	In between each calf	Between batches of calves	Once daily/twice daily/after every feeding session	Other	N (%)
Individual			X		24 (13)
		X			6 (3)
	X				5 (3)
				X	2 (1)
	X		X		1 (0.5)
		X	X		1 (0.5)
Group/individual and group			X		90 (50)
	X				14 (8)
		X			14 (8)
	X		X		10 (6)
		X	X		6 (3)
				X	3 (2)
	X	X			2 (1)
	X	X	X		2 (1)
				Total	180

Of those that only feed calves individually, the majority of farmers cleaned equipment once or twice daily, six cleaned in between each calf, and seven farmers cleaned between batches of calves. One participant cleaned once per week, and another did not clearly state their response.

For herds where calves were housed in groups, or where there was both individual and group housing, the most common frequency of cleaning was once or twice daily. Twenty-four farmers reported that they cleaned feeding equipment between each calf, and 28 clean between batches of calves. Of the three farms that could not be categorised, the responses were: cleaned every few days, every second day, and one farmer did not clearly state their response.

3.3.8 Mycoplasma bovis in the herd

When farmers were asked if they were aware of *M. bovis* prior to participation in the BTM study, 82% were aware and 18% were not (Table 3-19). One participant did not answer the question.

Four percent of farmers used a vaccine against *M. bovis* and the remaining participants had not. Of those who have used vaccines against *M. bovis*, five used the American import Myco-B One-Dose™ vaccine (American Animal Health, Grand Prairie, Texas, USA), and two used an autogenous vaccine specific to the herd.

Table 3-19: Questionnaire responses: questions relating to farmer awareness of Mycoplasma bovis and vaccine use

Question number	Question topic	Responses	N* (%)
Q14	Farmer awareness of *M. bovis* prior to study	Aware	147/180 (82)
		Not aware	33/180 (18)
Q16	Use of *M. bovis* vaccine	Yes	8 (4)
		No	174 (96)

*Denominator is 181 unless stated otherwise

Participants were then asked what the history of their herd was regarding *M. bovis* (Table 3-20). Two participants did not answer this question. The majority of participants stated that to their knowledge *M. bovis* had never been present in their herd, and around 15% of the farmers had never considered that *M. bovis* could be present in their herd. One quarter of participants that suspected *M. bovis* could be present in their herd, and only sixteen percent of farmers reported that *M. bovis* was previously or currently present with a diagnosis by a vet.

Table 3-20: Responses to Q15: history of Mycoplasma bovis in the herd

Currently present, confirmed diagnosis	Previously in herd, confirmed diagnosis	Farmer or vet currently and/or previously suspect, no confirmed diagnosis	To knowledge never present	Never considered could be in herd	N (%)
			X		57 (32)
		X			44 (25)
				X	26 (15)
	X				22 (12)
			X	X	13 (7)
X					6 (3)

Currently present, confirmed diagnosis	Previously in herd, confirmed diagnosis	Farmer or vet currently and/or previously suspect, no confirmed diagnosis	To knowledge never present	Never considered could be in herd	N (%)
		X	X		5 (3)
	X	X			3 (2)
X	X	X			2 (1)
X	X				1 (1)
				Total	179

3.4 Results – Calf seroprevalence study

3.4.1 Participant demographics

In total there were 36 dairy farms throughout Scotland that participated in the calf study. These farms represented 20% of the calf population in the BTM prevalence study, and 4% of the total population of dairy farms in Scotland (Table 3-21). Most participants were located in Dumfries and Galloway (31%, 11/36), Ayrshire (22%, 8/36) in Ayrshire, Lanarkshire (11%, 4/36) and Argyllshire (11%, 4/36).

Table 3-21: Distribution of herds in the calf study in Scotland by region

Region	N (%)
Aberdeenshire, Angus & Moray	1 (3)
Ayrshire	8 (22)
Argyllshire	4 (11)
Stirlingshire & Clackmannanshire	2 (6)
Perth & Kinross	1 (3)
Fife	2 (6)
Dumfries & Galloway	11 (31)
Highlands, Orkney & Shetland	0 (0)
Dunbarton & Renfrew	1 (3)
Lanarkshire	4 (11)

Region	N (%)
Lothian	2 (6)
Scottish Borders	0 (0)
Total	36

3.4.2 Herd structure

The number of 4-8 month-olds ranged from 10 to 121, with a mean of 45 (Interquartile range (IQR) = 23.5). The number of 10-14 month-olds ranged from 6 to 108 with a mean of 42 (IQR = 24). The median number of animals in both age groups was 40.

3.4.3 Calving management

The majority of herds use both AI and a breeding bull, 38% use AI only, and 3% use a breeding bull only (Table 3-22). Only 25% of farmers had purchased a breeding bull.

When a cow begins calving, 69% of farmers will calve the cow in a group pen and then move her to an individual pen and the remaining farmers move the cow to an individual pen when she starts calving. The majority of farmers used straw bedding in their calving pens, sand and paper bedding were both used by 3%, and 6% of farmers used a combination of sand and sawdust (Table 3-22).

Table 3-22: Questionnaire responses: questions relating to breeding and calving

Question number	Question topic	Responses	N* (%)
Q18a	Breeding	Breeding bull	1/34 (3)
		Artificial insemination	13/34 (38)
		Both	20/34 (59)
Q16	Calving cows	Move to individual pen when she begins calving	11 (31)
		Calve in group pen then move to individual pen after	25 (69)
Q18b	Bought a bull	Yes	9 (25)
		No	27 (75)

Question number	Question topic	Responses	N* (%)
Q17	Bedding in calving area	Straw	30 (83)
		Sand	1 (3)
		Paper	1 (3)
		Sawdust	2 (6)
		Straw & sawdust	2 (6)

*Denominator is 36 unless stated otherwise

3.4.4 Calf housing management

Farmers answered various questions relating to the housing of calves (Table 3-23). The number of calves that were housed per pen was an open-ended question, therefore responses were categorised into <12 calves/pen, >12 calves/pen, and both. The number of calves per group is a proxy for larger group pen housing and mixed age range housing (C. Mason, Personal Communication). Larger groups suggest mixed age ranges, which in turn increases the risk of disease spread. Fewer farmers housed <12 calves in a pen, the majority housed >12 calves in a pen, and some had a combination of both.

The age range within calf pens was also an open-ended question, and responses were categorised into <1 month and >1 month. Almost three quarters of farmers housed calves together with more than one month of an age gap.

Only three farmers housed calf pens in different airspaces, and five farmers housed all pens in the same airspace. The majority of farmers had only some pens in the same airspace. In most of the herds, there was opportunity for nose-to-nose contact between some of the calf pens. A small number of herds had shared water troughs between all calf pens, 33% had sharing between some pens, and the majority of herds had separate water troughs for every pen.

When asked if the lactating herd and youngstock were housed in the same airspace, the majority of herds housed the two groups in different sheds or farms, and only 8% housed their cows and calves in the same shed.

Table 3-23: Questionnaire responses: questions relating to calf housing

Question number	Question topic	Responses	N* (%)
Q3	Number of calves/pen	< 12	8/35 (23)
		> 12	17/35 (49)
		Both < 12 and > 12	10/35 (29)
Q4	Age range within pens	< 1 month	9/35 (26)
		> 1 month	26/35 (74)
Q5	Airspace of calf pens	All pens	5 (14)
		Some pens	28 (78)
		No pens	3 (8)
Q6	Opportunity for nose-to-nose contact between pens	All pens	3 (8)
		Some pens	30 (83)
		No pens	3 (8)
Q7	Sharing of water troughs between pens	All pens	3 (8)
		Some pens	12 (33)
		No pens	21 (58)
Q15	Housing of lactating herd and calves	Same shed/air space	3 (8)
		Different shed/air space	28 (78)
		Different farms/sites	1 (3)
		Different shed/air space and on different farms/sites	4 (11)

*Denominator 36 unless stated otherwise

3.4.5 Calf feeding practices

In response to Q10a, all farmers fed cows' colostrum to every calf (Table 3-24). Farmers always fed cows' colostrum to calves and never replaced it with

artificial colostrum (Q10b). Most participants fed calves colostrum from their own mother, and 8% fed colostrum from another cow in the herd. Thirty-one percent of farmers fed a combination of the two. Only one participant reported feeding pooled colostrum to calves. Just under half of the farmers never feed cows' milk to calves, whereas only 19% always feed cows' milk. Thirty-three percent of farmers will sometimes or very rarely feed cows' milk. Thirty-six percent of farmers fed cows' milk to all of their calves. A small number of farmers reportedly fed cows' milk to their dairy bull and beef calves only. Only one participant pasteurised cows' milk before feeding calves (Q12c). The time at which farmers reportedly stopped feeding cows' milk and fed only artificial milk varied with a small number of farmers stopping after the 1st day of life, 20 farmers switched to artificial milk between 2-7 days of a calf's life, and 11 farmers changed after eight or more days (Q13). Only two farmers had completely separate staff who worked with the milking herd and calves, and eight reported that they usually had separate staff who sometimes had to work with both age groups. Most farmers did not have separate staff.

Table 3-24: Questionnaire responses: questions relating to calf feeding practices

Question number	Question topic	Responses	N* (%)
Q10a	Feed cows' colostrum to calves	Always	36 (100)
Q10b	Calves fed cows' colostrum	All	36 (100)
Q11	Colostrum source	Dam	21 (58)
		Another cow in the herd	3 (8)
		More than one cow in herd (pooled colostrum)	1 (3)
		Dam & another cow in the herd	11 (31)
Q12a	Feed cows' milk to calves	Always	7 (19)
		Sometimes	8 (22)
		Very rarely	4 (11)
		Never	17 (47)
Q12b		All	13 (36)

Question number	Question topic	Responses	N* (%)
	Calves fed cows' milk	NA	18 (50)
		Dairy bulls & beef calves	3 (8)
		Beef	2 (6)
Q12c	Pasteurise cows' milk	Yes	1/19 (5)
		No	18/19 (95)
Q13	Stop feeding cow milk	After 1st day	5 (14)
		2-7 days old	20 (56)
		8+ days old	11 (31)
Q14	Milking and calf rearing staff	Completely separate personnel for cows and calves	2 (6)
		Usually separate staff but sometimes have to work with both	8 (22)
		Same staff work with cows and calves	26 (72)

*Denominator is 36 unless stated otherwise

3.4.6 Mycoplasma bovis in the herd

Only three farmers had cases of *M. bovis* diagnosed in their herd, one by post-mortem, one by a post-mortem and blood sampling, and one by nasal swabbing and blood sampling.

Half of the farmers reportedly treated 5-25% of their calves with antibiotics and one quarter treated 5% or less (Table 3-25). Eleven percent treated 25-50% and 14% of farmers treated 50-100%.

Table 3-25: Questionnaire responses: questions relating to antibiotic treatment and Mycoplasma bovis in the herd

Question number	Question topic	Responses	N* (%)
Q8	Antibiotic treatment	0-5%	9 (25)
		5-25%	18 (50)
		25-50%	4 (11)
		50-100%	5 (14)
Q9	*M. bovis* cases	Yes	3 (8)
		Post-mortem	1/3 (33)
		Post-mortem & blood sample	1/3 (33)
		Nasal swab & blood sample	1/3 (33)
		No	33 (92)

*Denominator is 36 unless stated otherwise

3.5 Discussion

The findings of this study provide a good general, and up-to-date, overview of the management practices and structures of dairy farms in Scotland. To the authors knowledge, this is the largest survey of Scottish dairy farm management practices in recent years.

3.5.1 Participant demographics and herd structure

Based on the geographical distribution of participants in the bulk milk prevalence study, the author felt that the dairy farm population in Scotland was well represented. The main dairy farming regions are in the southwest of the country, Dumfries and Galloway, Ayrshire and Lanarkshire where the climate and topography supports grass growth.

In 2021, the average size of dairy herds in Scotland was 209 cows (SDHA, 2021). According to the results of the BTM study questionnaire, there was an almost even split of herds that had less than 200 cows and those that had more than 200 cows. Information on the herd sizes in Scotland was difficult to obtain and access to full datasets was restricted due to data protection regulations. Some

data was available from the Scottish Dairy Herd Analysis (SDHA, 2021, 2020), however only the average herd sizes by region were published. Therefore, comparisons could not be drawn between the distribution of herd sizes for the total dairy herd population in Scotland and the study herds.

As the average herd sizes provided by the SDHA were grouped by region, the study herds were also grouped by region using the farm addresses. What became apparent was that there were some discrepancies as to the defining boundaries for each region. Postcodes were used to assign farms to a region. For some regions such as Aberdeenshire, it was easy to assign farms to this region based on their address and postcode. Others were not as straight forward, which resulted in a number of regions being combined, for example Dunbartonshire and Renfrewshire. Additionally, there were many smaller sub-regions that could be combined to create larger regions. An example of this is that there were three sub-regions in the SDHA data: Wigtownshire, Kirkcudbrightshire, and Dumfriesshire, all of which are within the overall region of Dumfries and Galloway. There were some smaller regions in the SDHA data that contained less than five farms, therefore these regions were grouped with others to ensure confidentiality of study herds was maintained.

Over the past couple of decades, the number of dairy herds in Scotland has declined while the average dairy herd size has steadily increased (Uberoi, 2021). A recent study on Scottish dairy herds reported that 51% of farms had increased in size from 2015 to 2022, and 33% were planning to increase in the future (Shortall and Lorenzo-Arribas, 2022). The majority of farmers in the present study were maintaining the size of their herd. Farmers were not constrained to a time-period when asked if they were changing, or had recently changed, the size of their herd. It was assumed that farmers responded based on whether they were actively changing their herd size or not. Just over half of the farmers had purchased at least one animal within the last 12 months. A higher proportion of farmers bought in 12-24 month-olds rather than 0-12 month-olds, which makes sense as they were likely purchasing replacement heifers. Buying in asymptomatic carrier animals is one of the main methods of introducing disease into a dairy herd (Sayers et al., 2013). Many diseases including *M. bovis*, BVD virus, and leptospirosis can easily be introduced via carrier animals. This is why it is imperative for farmers to test and quarantine all newly purchased animals.

Maintaining a closed herd does not necessarily prevent the introduction of disease by other means such as via semen for AI (Haapala et al., 2018), however it significantly reduces the risk of introducing disease.

At each of the four sampling points, the percentage of herds that had all cows contributing to the BTM at the time of sampling, i.e. no sick cows nor any receiving treatment, ranged from 17-25%. This meant that between 75-83% of herds had at least one sick cow at each sampling point which would be expected.

One farmer reported that they had 50 bulls on farm, which is significantly higher than the other participating herds. This herd had over 1,000 cows which was considerably larger than most herds in the study (the mean and median number of cows in the study was 245 and 200, respectively). With such a large number of cows, this herd may have needed more breeding bulls than the average herd. Another possible explanation could be that the farm had a breeding facility.

Only 12 farmers did not rear dairy bull and beef calves, though farmers were not asked what they do with these calves. Due to the low value of dairy bull calves, euthanasia shortly after birth was previously a common occurrence (Mahendran et al., 2022). The industry shift away from the euthanasia of dairy bull calves has resulted in more farmers opting to rear dairy bull and beef calves on their farm or send them to a rearing unit.

In the present study, just over 20% of herds sold their dairy bull and beef calves before weaning, which is considerably lower than a recent UK wide study on calf management practices in which it was reported that over 60% of the herds sold dairy bull and beef calves prior to weaning (Mahendran et al., 2022). In the same study, just over one quarter of the farmers sold these calves after weaning, whereas in the present study the majority of herds sold their dairy bull and beef calves after weaning. One possible explanation for a much lower proportion of herds in this study selling calves prior to weaning could be due to the difference in participant selection criteria. Mahendran et al. (2022) targeted herds that rear their own replacement heifers. Rearing replacements on farm will require more space to allow for a higher number of calf rearing pens. Consequently, this means that there is less space on farm for rearing dairy bull and beef calves. In the present study, this was not a criterion for selection and as such, 10% of herds

in this study bought in 0-12 month-olds and 58% bought 12-24 month-olds in the last 12 months. It could be that in this study, there was a combination of herds rearing replacements and selling dairy bull and beef calves earlier, and herds that don't rear replacements (or not as many) and so have the space to rear dairy bull and beef calves for longer.

Around one quarter of farmers reared their dairy bull and beef calves until slaughter. The age at which these calves are sent to slaughter was not specified, therefore they could be sent before or after weaning. In the UK, the majority of dairy bull and beef calves will be sold at slaughter weight, and the age at which youngstock reach slaughter weight will vary between farms. If calves are retained for longer before slaughter, there is the increased risk of disease spread due to the potential mixing of age groups within pens.

3.5.2 Data collected at each sampling point

Between samples one and two, farmers were sent their sampling kit ahead of the time that their samples should have been collected and provided with guidance on when the samples were to be collected. Samples were to be collected roughly three months apart. Many farmers sent their samples back straight away which resulted in a shorter timeframe between the two sampling points rather than three months. For the third sampling point, farmers were sent their kits slightly closer to the time of sampling to increase the length of time between samples two and three. The median number of days between samples two and three was considerably higher than the time between the other samples. It was challenging to get farmers to return their second BTM samples for testing, which caused the large gap between samples one and two. This could be explained by the time of year in which the samples were being collected as sample points two and three ran over the spring and early summer months. This is a busy time for herds that calve during this time which could have resulted in farmers forgetting to collect samples or not having the time to collect them. Additionally, if herds also had a flock of sheep, then they would likely be lambing around this time, which may also have had an impact on the timing of the sampling.

On several occasions farmers misplaced their sampling kits and a new kit had to be posted to the farm. To minimise dropout and to ensure farmers sent in their next samples, regular contact was maintained with participants, sending reminders when they were expecting new kits, and to encourage them to send back their samples and project forms. This work was carried out by only one researcher, and although strict records of contact dates were kept, there is always the potential for a margin of error.

Somatic cell count

Measuring the SCC of bulk milk is a general indicator of herd-level udder health, and an indirect measure of milk quality (Schukken et al., 2003). A SCC of ≥200,000 cells/ml is considered to be high and often associated with the presence of bacterial intramammary infections (Bradley and Green, 2005). In the UK, there are a variety of pathogens that can cause mastitis (AHDB, 2021). Although *M. bovis* is one of the potential mastitis-causing pathogens, in the UK it is not a primary cause of mastitis and therefore a high BTM SCC is likely to be caused by another pathogen (Timonen et al., 2017). In the present study at least 84% of the participants had a SCC of less than 200,000 cells/ml at each of the four sampling points. Participation by farmers in research studies can be biased as high producers with low somatic cell counts are more likely to participate, whereas low producers with high somatic cell counts are less inclined to participate due to fear of judgement (Bauman et al., 2018). Farmers with good mastitis management and a low number of cases may have been more inclined to participate than those with recurring mastitis challenges.

The UK BTM SCC often increases at the time of turnout in April/May until housing around October (Green et al., 2006). This seasonal increase in SCC was not observed in the BTM study, however the collection of more than four BTM samples may have been required to see this trend.

Thirty-five percent of farmers did not report their most recent SCC with their first BTM sample. To try to minimise response burden by reducing the number of sheets of paper the farmers had to go through, the sample submission form was printed on the back of the letter addressed to farmers. As a result, it was speculated that this form was overlooked by farmers as it was not printed on a separate sheet of paper. The percentage of farmers that did not complete their

sample submission form in the subsequent sampling points was below 10%. In these sampling kits the sample submission form was printed on a separate sheet of paper to ensure that farmers would not miss the form.

Cows contributing to the BTM

Between 84-87% of the total lactating herd contributed to the BTM samples at each sampling point. This high proportion of the herd contributing confirms that the majority of lactating cows in the study herds were represented in the BTM samples. This is what would be expected on a typical UK dairy farm and is in line with previous figures. Two hundred and forty-two herds were included in an analysis of key performance indicators for mastitis in UK dairy herds (Hanks, Taylor and Kossaibati, 2024). Between 2016 and 2023, the percentage of cows that did not have mastitis during the entire lactation increased from 79% to 85%, respectively. Furthermore, in 2023, the average number of clinical mastitis cases was 22 cases per 100 cows. This shows that in typical UK dairy herds, the level of clinical mastitis remains relatively low.

The calving pattern will largely influence this figure. When sampling in a block calving herd, if the samples were collected prior to calving, there would be a smaller percentage of cows contributing to the BTM, whereas if sampled later in the calving block, most, or all cows will have contributed to the BTM. In year-round calving herds, the percentage of cows contributing to the BTM should not change much throughout the year as cows will be calving continuously. Of the herds that were outliers in this data (had lower proportions of the herd contributing to the BTM), some of them were block calving herds and the timing of sample collection occurred just before calving at the time when the cows would be dried off. Though, there were also a number of herds with a low proportion of the herd contributing to the BTM but were year-round calving herds.

The herd size data recorded from Q1 of the BTM study was compared to the number of cows recorded at each sampling point. This comparison provided an internal validation of the cow numbers provided by the farmers at each of the timepoints. There were some instances where the farmers misunderstood the form and under ‘cows not contributing due to illness or treatment’ wrote a figure which appeared to be the total number of cows that contributed plus the number of sick cows, rather than just the number of sick cows. Also, in some

farms, the differences between the submission form cow numbers and Q1 cow numbers were >180. The reasons for such large differences in cow numbers could be due to a variety of reasons. Firstly, there is a risk that the farmer provided the wrong figure. Secondly, the farmer could have been changing the size of the herd, for instance, increasing the herd by retaining replacements bred on farm or by purchasing new cattle. Alternatively, if the farmer is downsizing, they could have sold stock. Cows may have been culled from the herd due to various other unknown reasons which would decrease the total number of the lactating herd. Finally, if any farmers manage their herd across multiple farms, they could have moved some of the herd onto or off the study farm, which would result in a difference in the total cow numbers.

3.5.3 Calving management and breeding bulls

From the questionnaire, only 22% of the BTM study herds had a breeding bull on farm at the time of data collection, and of those that did have a bull, the mean number of bulls on farm was 2. After being made available in the UK in 1942 (Brassley, 2007), AI was used on around 60% of all cows in England and Wales (Wilmot, 2007). AI is still widely used within the UK dairy industry due to its high success rate (Vishwanath, 2003), low costs, control of venereal diseases, and simplicity (Howley et al., 2012). This method of breeding enables farmers to introduce new and improved genetics while still maintaining a closed herd. Another reason that may encourage farmers to choose AI over a breeding bull is the risk of injury associated with keeping a bull on farm.

Most farmers that participated in the calf study used a combination of AI and a breeding bull. In these herds it is likely that cows undergo AI once or twice and any cows that fail to come into calf will then be served by the bull, often called a ‘chaser’ or ‘sweeper’ bull. Three percent of farmers used a breeding bull only, which reflects the high uptake of AI throughout the UK dairy industry.

Fewer farmers used separate calving pens for cows according to the responses in both questionnaires. This finding is contrary to that of Brown et al. (2021) who reported that separate calving pens were used on 73% of Northern Irish farms. Moving a cow to a separate pen when she begins calving minimises the risk of direct disease transmission from other cows in the herd to the newborn calf

(Gille et al., 2018) or indirect transmission through contact with faeces, dust, or feed (Donat et al., 2016; Doré et al., 2012). It also enables farmers to pay attention to a cow as she is calving and create a safer environment to work in. Farmers may not use a separate calving pen due to a range of reasons. The number of pens required to house separate calving cows would be high and many farms do not have the capacity to do so. Furthermore, someone would need to always be nearby to move cows just before or as she begins to calve. Cows do not like being housed alone, if they are moved into a separate pen prior to calving then this can reduce feed intake. Alternatively, if cows are moved to an individual pen during calving, this can slow down the calving and risk issues (Proudfoot et al., 2013).

The majority of study herds operated on a year-round calving pattern (87%), which was comparable to the UK dairy farm population where 79% calve year-round (AHDB Dairy, 2017).

The introduction of new heifers into the lactating herd can affect the BTM quality. Heifers are more susceptible to mastitis (Barkema et al., 1998; Fox et al., 1995) which increases the presence of pathogens in the BTM and the SCC (De Vliegher et al., 2004). In herds that calve all year, there will be a continuous input of heifers into the lactating herd, therefore variation in the BTM will occur throughout the whole year. Whereas in block calving herds, there will be a high concentration of new heifers entering the lactating herd within a short period of time. This will increase the BTM SCC and the presence of pathogens. During periods where there are no new heifers entering the lactating herd, the quality of the BTM may be improved and there will likely be a reduced pathogen load.

The impact of calving pattern on the results of samples taken from calves may be similar to the impact on BTM samples. In block calving herds, calves are born in a smaller timeframe, thus there will be larger groups of calves of similar ages that have the same management requirements. However, as there will be a larger number of calves within the herd during the calving block, there is more opportunity for disease spread, which could in turn increase the level of pathogens within the calves during that time.

In year-round calving herds, calves will be born intermittently with periods where only one or two arrive at the same time. Consequently, there will be a

variation in the conditions and challenges that calves are exposed to at different times of the year. The health and immune responses of calves will likely be inconsistent throughout the year, resulting in a variation in the prevalence of different pathogens. Furthermore, these few calves may receive less attention compared to those in block calving herds, where larger groups of calves are managed together, and farmers may hire more staff during peak calving periods. This could lead to a higher level of disease among calves in year-round calving herds.

Overall, the calving pattern causes fluctuations in the types and prevalences of pathogens in both the BTM and calves, consequently influencing the results of diagnostic testing carried out throughout the year.

3.5.4 Feeding practices

Colostrum

In utero transmission of immunoglobulins from cow to foetus does not occur due to the placental barrier between the maternal and foetal blood supplies (Quigley and Drewry, 1998). Therefore, it is imperative that calves receive passive immunity via colostrum immediately after birth to protect them from infectious diseases (Uetake, 2013; Weaver et al., 2000). In the BTM study, farmers either fed cows' colostrum alone, or a combination of both cows' colostrum and artificial colostrum. In the calf study, when farmers were asked how often cows' colostrum is fed to calves, 100% reported that they always feed it, and that all calves receive cows' colostrum.

Depending on the calving pattern, a farm may go through periods of time with inadequate supplies of colostrum (Godden, 2008). When this occurs, to ensure that all calves receive passive immunity, farmers will generally feed an artificial colostrum as a replacement.

When asked what cows they source their colostrum from, over half of the farmers fed colostrum from the dam to the calf. This is likely the most convenient source of colostrum, particularly if the calves are left to suckle rather than milking the cow and feeding the colostrum via bottle or tube. There

is also reduced risk of exposing the newborn calf to disease from other cows within the herd.

A small proportion of farmers reported that they source colostrum from another cow in the herd, and over thirty percent of farmers sourced colostrum from the dam and another cow in the herd. Sometimes a cow may produce poor quality, or very low quantities of colostrum. Older cows tend to produce higher quality colostrum likely as they have been exposed to more farm-specific pathogens (Pritchett et al., 1991). If a calf is born to a first time calver, the farmer may be inclined to source colostrum from another cow in the herd with the belief that the colostrum will be higher in quality.

Very few farmers fed pooled colostrum to calves. This is contrary to a recent study published by Denholm et al. (2023), who reported that just under half of dairy farmers from across the UK fed pooled colostrum to calves. This method of feeding should generally be avoided as high-quality colostrum may be diluted by low quality colostrum and can increase the risk of exposing calves to colostrum-borne pathogens (Godden, 2008; Weaver et al., 2000). Although the risk of disease transmission is increased, farmers may pool colostrum for convenience when feeding calves, particularly if they rear a large numbers of calves (Denholm et al., 2023).

Milk

Over 60% of participating farmers fed only artificial milk to calves. This finding is similar to that of Mahendran et al. (2022), who reported that 52.8% of dairy herds throughout the UK fed artificial milk, and likewise a study of Northern Irish dairy herds reported that 81.8% of farmers fed artificial milk (Brown et al., 2021). One of the benefits to feeding artificial milk is that calves are not exposed to diseases such as *Mycoplasma bovis*, Johne's disease, and *Salmonella spp.* from the lactating herd through the milk. Some farmers choose not to feed artificial milk to calves as it is expensive to purchase, and they can save money by feeding waste milk. Ten percent of farmers fed only cows' milk to calves, which was similar to that of a study of Norther Irish herds where 18.2% of herds fed only cows' milk (Brown et al., 2021).

Herd membership to farm assurance schemes was not included in either questionnaire. Around 98% of the UK milk supply comes from Red Tractor Assured farms (C. Mason, Personal Communication), which stipulates that calves are not to be fed waste milk and reflects the large proportion of participants that reportedly fed only artificial milk to calves.

Waste milk is milk that is discarded as it is not fit for human consumption as it comes from cows with high SCC or cows that have received antimicrobial treatment and are still within the product's withdrawal period (AHDB Dairy, 2020). Farmers are advised against feeding waste milk to calves as it increases the presence of resistant bacteria in respiratory tracts and the gut of calves (Maynou et al., 2017). However, many farmers still choose to feed waste milk alone or in conjunction with artificial milk (Brunton et al., 2012). In both questionnaires, farmers were not asked to distinguish between fresh bulk milk or waste milk.

Some farmers may not wish to disclose that they fed waste milk to calves for fear of judgement and may have provided inaccurate answers that reflect what they wish to do rather than what they actually do (Scholl et al., 1992). Consequently, it was decided to offer three response options: artificial, cows', or both, where cows' milk could be interpreted as fresh bulk milk and/or waste milk.

Just over one quarter of farmers fed a combination of cows' and artificial milk to calves. With many milk buyers basing their pricing off the volume of milk, it is unlikely that farmers would opt to feed fresh bulk milk and would rather utilise waste milk at no additional cost or use artificial milk. During periods of time where there are a higher number of cows with high SCCs or antimicrobial withdrawals, farmers have a plentiful supply of waste milk to feed to calves and they won't need to supplement with artificial milk. If there are few cows producing waste milk, artificial milk may be required to ensure all calves are fed. Likewise, during the summer months when there is a flush of milk production in summer grazing herds, artificial milk will not be required, whereas in the winter months milk production will be lower, and farmers may need additional artificial milk. These changes in the frequency of feeding cows' milk

were reflected in the findings from the calf study where 22% of farmers sometimes and 11% very rarely fed cows' milk to calves.

Some farmers may feed groups of calves differently, for example, 36% of farmers fed cows' milk to all calves, 8% fed cows' milk to dairy bull and beef calves, and 6% of farmers fed cows' milk to beef calves only. While the immune system of young calves is developing, farmers may decide to feed artificial milk to younger calves and waste milk to older calves. Conversely, as shown in the results of the calf study, farmers may start by feeding cows' milk and switch to artificial milk after the first day, within the first week, or beyond one week of age.

Pasteurisation

Very few farmers pasteurised cows' milk and colostrum prior to feeding calves. This finding is similar to that of Denholm et al. (2023) who reported that under 10% of 248 dairy farmers throughout the UK pasteurise cows' colostrum. Pasteurisation can damage immunoglobulin in colostrum, however heat treatment for a longer period of time with a lower temperature will kill pathogens while maintaining IgG levels (Donahue et al., 2012; Godden et al., 2019). As *M. bovis* can be spread via cows' milk and colostrum to calves, pasteurisation kills the pathogen, prevents transmission in milk and colostrum, and thus reduces the need for antibiotic treatment (Maunsell et al., 2012).

While the advantages of pasteurising cows' milk and colostrum are recognised, practical and economical barriers continue to limit its widespread uptake throughout UK dairy farms. The reluctance of farmers to adopt pasteurisation is mainly due to the initial investment in the pasteurisation equipment, as well as the ongoing costs of maintaining and operating the equipment (Godden, 2007). This will be a particular hinderance for smaller farms where it may not be feasible. The impact of pasteurisation is not always visible straight away and can be more subtle. For example, pathogens such as *M. bovis* can be endemic in a herd causing coughs and head tilts in calves. After the introduction of pasteurisation, there may be a gradual improvement in the general health and immune functioning of the calves. The improvements may not be 'visible' to the farmers straight away, which may discourage them from adopting pasteurisation. Furthermore, pasteurisation requires time and additional labour, which may not

be attainable in most farms, particularly as it is difficult to find and retain farm staff in the UK (Nye, 2021).

Pre-weaned calves

The majority of farmers reported that they group feed pre-weaned calves. This finding is contrary to that of Brown et al. (2021) who found that most Northern Irish farmers in their study used single teat feeders. The authors reported that this may be related to the fact that on a large proportion of these farms, calves were not grouped until they were at least one week of age. In the present study, farmers were not asked when they group calves, only if they group them.

The method in which calves are fed is highly dependent on the housing setup. Where calves are group housed it is more practical for calves to be fed from a group feeder, whereas calves housed individually or in pairs will likely be fed by an individual bucket and/or teat.

When comparing responses to Q8 (housing of pre-weaned calves) and Q12 (method of feeding pre-weaned calves), the responses were varied. Of those who individually housed calves, 82% fed calves individually, and 42% of farmers who group housed calves also group fed them.

Most farmers that did a combination of individual and group housing also fed calves individually and in groups. In these herds calves would have been housed and fed individually for a period of time after birth and then grouped with other calves and fed in those groups.

Depending on the size of the herd and the number of personnel employed, the management of cows and calves may be carried out by separate staff. This will be more common in larger dairy herds where there is justification to employ calf rearers and milkers. Only 6% of herds in the present study had completely separate staff. Often Scottish dairy farms are run by a family or have very few extra staff, therefore it is not possible for employees to work with only one of the groups of cattle. A few farmers reported that they usually have separate staff who work with the cows or calves though sometimes the staff have to do both. This structure is likely to be very common throughout the UK dairy industry as there may be occasions where the farm is short-staffed or other occurrences

during the day take staff away from their usual duties (i.e. milking or calf rearing) which then has to be carried out by another member of staff.

Cleaning and disinfecting of calf feeding equipment

Regular cleaning and disinfecting of feeding equipment are an integral part of calf rearing. The immune system of a calf is naïve to the infectious pathogens that they may be exposed to on farm, which is why many farm assurance schemes require farmers to ensure that calf feeding equipment is kept in a clean condition (Assured Food Standards, 2023).

When it comes to feeding calves, the gold standard is to clean and disinfect feeding equipment after each use to prevent the spread of disease, though this is not always practiced on farm (Mahendran et al., 2022). This is particularly important where calves are group fed, or feeding equipment is shared among individuals (Brown et al., 2021). In the present study, it was difficult to identify what could be classed as ‘good’ or ‘bad’ practice due to the wording of the question. Cleaning and disinfecting feeding equipment daily will reduce the risk of disease spread, however, if this is only done once daily and equipment is shared between calves, there would still be the possibility for infection to spread.

It became apparent that the question asking farmers how often they clean and disinfect calf feeding equipment was too vague. This was a multiple-choice question with the response options being: in between each calf, between batches of calves, once daily, twice daily, and every other feeding session. There was the option to select ‘other’ where participants could input responses not listed. Once data collection had begun, it was evident that the pre-stated response options caused some confusion to participants and could have been interpreted in a variety of ways. The wording of this question resulted in a number of participants selecting ‘other’ and writing that they clean but do not disinfect, and vice versa. Similarly, some farmers stated that they cleaned and disinfected equipment between each calf, this could be interpreted as either: clean equipment is provided to each calf daily, or the bucket/teat is only cleaned when it is given to a different calf, which may not be daily.

Additionally, as the questionnaire was multiple choice, participants selected a variety of combinations which made it challenging to categorise the responses in a succinct way. To reduce response burden to farmers, the questionnaire was made as short as possible, however this may have resulted in some questions being combined which should have preferably been two separate questions. Question 13 is a good example of this as there should have been one multiple choice question on cleaning of feeding equipment and another on disinfecting feeding equipment. Alternatively, an open-ended question could have been included asking farmers to describe how often they clean and disinfect the calf feeding equipment. However, the responses may have been equally as challenging to interpret as there could be great variation in cleaning and disinfecting practices between farms.

3.5.5 Housing

Lactating herd

The most common housing practice was housing of the lactating cows for part of the year, similar to a more recent study on farmer practices in Scotland who reported that 41% of farmers house cows seasonally (Shortall and Lorenzo-Arribas, 2022). Housing cows indoors during the winter months when the climate is less favourable for grazing, then grazing in spring and summer has, for the most part, been the traditional method of housing dairy cows throughout the UK (Haskell et al., 2006; March et al., 2014). Only 3% of study herds operated on a maximum grazing system, in which cows are grazed outside for most or all of the year. This finding is consistent with a study on farming practices in the UK where on less than 1% of dairy farms, cows were housed outside all year round (March et al., 2014).

Over one quarter of farmers housed some or all of their cows permanently, which is also known as zero grazing, similar to recent studies that reported 19% (Shortall and Lorenzo-Arribas, 2022), and 16% (March et al., 2014) of farmers housed cows all year round. Previously, cows were generally housed permanently in areas where the land could not support grazing, however irrespective of the quality of land, zero grazing is becoming increasingly popular. Aside from public perception of permanently housing cows (Taverner, 2015), many farmers believe

that cows should access pasture for some part of the year (Shortall and Lorenzo-Arribas, 2022). When asked why cows were housed permanently, the most important factors were related to feeding high energy density feed to increase production, the use of a robotic milking system, and the distance for the cows to walk to the parlour for milking (Shortall and Lorenzo-Arribas, 2022).

Pre- and post-weaned calf housing

The housing environment for a pre-weaned, milk-fed calf significantly influences their growth and future productivity. Various types of calf housing are used throughout UK including individual hutches, group hutches, large pens within the same shed, and outdoor rearing (Brown et al., 2021). The structure of calf housing is highly dependent on a range of factors including the calving pattern, size of the herd, available space, farm staff, and milk buyer policies.

The type of calf housing was addressed in both studies. The BTM study focused on mixing of calves in groups at pre- and post- weaning. In the calf study, the grouping of calves was addressed in further detail, focusing on the age range, number of calves within pens, and the opportunity for contact between pens.

Although there is a greater risk of disease transmission between calves that are group housed, the welfare of the calves is higher and they also have improved solid feed intake (Brown et al., 2021). In the UK, grouped or paired housing of calves at birth or within the first few weeks of life is now required by many milk buyers (Mahendran et al., 2022). Furthermore, grouping calves by the age of eight weeks of age is a requirement by Scottish legislation (The Welfare of Farmed Animals (Scotland) Regulations 2010 (S.S.I. 2010 No. 388) Schedule 4) unless they are receiving veterinary treatment.

When it came to post-weaned calf housing in the BTM study, mixing of groups of calves occurred in the majority of herds. In many farms, this may have been the most convenient option to improve calf management due to the space, resources and staff available (Brown et al., 2021). These factors may also influence the number of calves that are housed in each pen.

Calves were housed in groups with less than a one-month age gap in only one quarter of study farms. Separating calves into age group and maintaining those

groups can be easier in herds that calve in blocks as there will be large numbers of calves around the same age (Cummins et al., 2016). This is also true for large herds, regardless of the calving period, as they will likely have multiple calves born every day. In herds that calve all year round, it may be more convenient to house calves with a wider age range in the same group as there will be a continuous flow of calves being born throughout the year. The disadvantage to year-round calving is that with a constant trickle of calves being born with no breaks does not allow farmers the time to properly clean and disinfect, increasing the risk of disease outbreaks (Palczynski et al., 2021). Furthermore, grouping calves with large age ranges results in young naïve calves being exposed to older calves (Nordlund and Halbach, 2019), and was shown to increase the risk of *M. bovis* seroconversion in fattening herds (Tschopp et al., 2001).

Calf rearing pens may be distributed throughout the farm, with pre-weaned, milk-fed calves housed nearer the parlour for milk and post-weaned calves in a different shed. In other systems, all youngstock may be housed within the same shed. Though, as mentioned previously, the available space in a farm will hugely determine the calf rearing setup. When it came to the housing of lactating cows and calves, most farmers housed the two groups of animals in separate sheds or air spaces. This is likely due to the availability of shed space and may also be for convenience.

Disease transmission can be facilitated where there are multiple adjoining pens with no solid partitions allowing opportunity for direct nose-to-nose contact, or shared water troughs between pens. Most farmers reported that they had some calf pens in the same air space, and also there was the opportunity for nose-to-nose contact between some pens. In most herds, calf pens had separate water troughs.

3.5.6 Mycoplasma bovis in the herd

Herd history

There was almost an even split of farmers who reported a combination of current diagnosed *M. bovis* disease, recent diagnosed *M. bovis* disease, and

suspected disease, and those who stated that there was no recent disease, nor they had never considered *M. bovis* could be present (Q15).

The response options stating that *M. bovis* had been currently or recently diagnosed were definitive, i.e. if *M. bovis* had been diagnosed by a vet, farmers could select those options. The responses from farmers who suspected *M. bovis* was present, or to their knowledge believed the disease was not present could have been subjective. The presence of symptoms such as pneumonia or a head tilt in calves, mastitis in cows, or lameness in any cattle, combined with a drop in milk production and failure of antimicrobial treatment could be suggestive of *M. bovis* in a herd (Nicholas et al., 2008). Identifying disease symptoms in cattle can be subjective, in that some farmers may spot the presence of particular symptoms which would make them suspect that the disease is present. Conversely, others may not notice certain symptoms and believe that a disease is not present when it is. Symptoms of *M. bovis* can be similar to those caused by a variety of other pathogens, for example in respiratory disease (*Mannheimia haemolytica* and *Pasteurella multocida*) and mastitis (*Escherichia coli* and *Streptococcus uberis*) (AHDB, 2021), which could make farmers suspect the presence of different diseases rather than *M. bovis*.

If there is history of a particular disease present in a herd, the farmer may have applied various changes to their management practices to reduce the risk of disease spread and to prevent reintroduction. There was no investigation into possible associations between herd history and the other questionnaire responses, except the farmers' awareness of *M. bovis* prior to participation. It could be speculated that farmers with diagnosed or suspected *M. bovis* disease may have adopted management practices that favoured within-herd biosecurity.

The purpose of Q15 was to get a feel for the history of *M. bovis* within the study herds and aimed to cover the main 'stages' of disease presence i.e. not present, possibly (suspected) present, and present (diagnosed by a vet), and at the same time capture the timeframe, i.e. previously vs currently. The farmers' interpretation of the multiple-choice options may have varied. The words 'previously' and 'currently' were used as it was hopefully clear that 'currently' referred to the present and 'previously' referred to the past. Though, the cut-off for 'previously diagnosed' vs 'currently diagnosed' were not defined, so

farmers were left to decide which best described their situation. For three farmers, both options were selected. In the same question, farmers had to select whether *M. bovis* had been diagnosed by a vet and/or was suspected by them or their vet. For most farmers, the question was likely straightforward to answer, especially if the herds clearly fit into one or more of the multiple-choice options. Confusion may have arisen not due to the wording, but rather the order of the multiple-choice options. In hindsight, it would have made sense to order the options from no disease to suspected disease and then diagnosed, when instead, the responses were in a random order.

Only two farmers did not answer this question, the reason for which is unknown. Had more farmers not answered the question, it could have suggested that the multiple-choice options were ambiguous. If any farmers did select the wrong options, particularly between diagnosed currently, diagnosed previously and suspected, for the statistical analysis there would be no risk of misclassification bias as these responses were grouped as one.

A minor change to the question could have been to split the 'suspected' option in the same format as the 'diagnosed' options so that farmers could select: 'I and/or my vet currently suspect…' and 'I and/or my vet previously suspected…'. Although this level of detail would be interesting to have, for the statistical analysis in the subsequent chapter, the responses would have been grouped to ensure that the group sizes were greater than 5.

This question could have been open-ended. At the time of developing the questionnaire, it was decided to minimise the number of open-ended questions as that style of question can be difficult to interpret and categorise.

No matter what changes could have been made to the question, it is likely that the responses to this question would have been grouped similarly, if not exactly the same, as they were.

Farmer awareness of *M. bovis*

A farmer's awareness of a particular disease may influence the management practices they adopt on their farm. Over 80% of participants were aware of *M. bovis* prior to participating in the BTM study, therefore the choice of

management practices and herd structures reported in this study may be more reflective of Scottish dairy farmers that are aware of this disease.

Use of vaccines against *M. bovis*

The use of vaccines against infectious diseases contributes towards the reduction of antimicrobial use in veterinary medicine (Gov, 2018). Where there is no, or limited, access to a licensed vaccine against a particular pathogen, autogenous vaccines are an alternative tool to increase disease resistance within a herd.

When the questionnaire in the BTM study was completed by participating farmers, vaccination options against *M. bovis* were limited in Scotland. At the start of the BTM study, eight farmers reported that they used a vaccine against *M. bovis*, six of which used the *M. bovis* bacterin vaccine Myco-B One-dose™. Uptake of the Myco-B™ vaccine in Scotland was steady at this time as it was available to use in the UK under a special import license. Although *M. bovis* was frequently publicised in farming and veterinary articles in the UK around this time, the disease was still considered new. Since the BTM and calf studies took place, another vaccine was developed, Protivity® (Zoetis, USA). This is the first modified live vaccine developed against *M. bovis* and as of November 2024, the vaccine became commercially available in the UK.

Only two farmers in the BTM study used an autogenous vaccine against *M. bovis*. These vaccines are formulated from the specific pathogen isolate that is present in one or several animals within the herd. Autogenous vaccines are herd-specific and can only be used in the herd that the pathogen isolate was obtained from. They can also be expensive which may be one of the reasons why uptake of these vaccines is minimal.

By using vaccines against *M. bovis*, immediately there will be a reduction in the incidence of clinical disease, including mastitis and arthritis in cows, and RD and otitis in youngstock (Dudek, Szacawa and Nicholas, 2021). Consequently, the overall health and productivity of the herd will increase as there will be an increase in growth rates of calves, milk yield and reproductive performance, and a decrease in mortality and morbidity, resulting in reduced costs of treatment and labour required. Furthermore, with increasing AMR, vaccines reduce the

reliance on antimicrobials for treating *M. bovis* (Ayling et al., 2000). Herd immunity can be attained with consistent vaccination over time.

There will be a reduced pathogen load and thus risk of transmission between individual animals and groups of animals.

Percentage of youngstock treated with antibiotics

Three quarters of the farmers in the calf study had treated less than 25% of calves with antibiotics within the last 12 months, and the remaining 25% of farmers had treated between 50-100%. This was a very small sample size of only 36 farmers therefore the results may not be comparable to antimicrobial use in the total dairy farm population in Scotland. As mentioned previously, farmers and veterinarians are encouraged to reduce their use of antimicrobials (prophylaxis and metaphylaxis), and only treat affected animals. Targeted antimicrobial use (AMU) is a particular focus in calf rearing in a bid to minimise respiratory disease. *M. bovis* is one of the major BRD complex pathogens. There are very few antimicrobials that are effective against it as most antimicrobial groups target structures that are not present in the pathogen (Calcutt et al., 2018). Consequently, it is better for farmers to identify the causative pathogen before treating with antimicrobials.

It must be noted that these figures are not contextualised, i.e., it is not known why the proportion of calves were treated in each herd. A low proportion of calves could have been treated as there simply was not much clinical disease in the herd, however, identifying symptoms requires farmer vigilance therefore despite a high prevalence of disease, it will go undetected by the farmer. A higher proportion of calves being treated could reflect a higher prevalence of disease, or it could be due to the use of prophylactic treatment.

It could be considered encouraging that the majority of farmers tested up to 25% of the calves with antibiotics, however the fact that one quarter treated between 50-100% shows that there is more work to be done on promoting good AMU.

Other topics not explored

There are other questions that farmers could have been asked in the study questionnaires relating to *M. bovis* within their herd. For example, in Q15 of the BTM study, if farmers selected 'I and/or my vet suspect *M. bovis* to be present in the herd', they were not asked why this was the case. This question could have been expanded with a second part asking farmers to either select from multiple-choice options or in an open-ended question explain what signs made them suspect *M. bovis* presence in their herd.

The presence of clinical *M. bovis* disease was not included in the BTM study as this was outside of the scope of the project. Furthermore, clinical symptoms of *M. bovis* can be difficult to distinguish as there are often multiple pathogens involved in the infection (Ridley and Hateley, 2018). Symptoms may also be less evident and in some cases infected animals are asymptomatic (Houlihan et al., 2007). Therefore, if farmers had been asked to state any clinical signs of *M. bovis* they observed, their responses may not have valid.

If this information had been captured, it could have been compared to the BTM test results and if the clinical signs were associated with the BTM antibody results, it would validate the farmers' suspicions.

Other questions relating to the productivity of the herd could have been explored, such as recent changes to the BTM SCC, the daily milk yield of individual cows, or the growth of youngstock. As discussed previously, an increase in the BTM SCC (specifically above 200,000 cells/ml) is an indication of mastitis within the herd. At each sampling point, farmers were asked to record their most recent BTM SCC, but they could have also been asked if there was a change in the BTM SCC in the last 12 months which could have indicated when *M. bovis* became more prevalent within the herd. The latter two examples of additional questions relate to individual animals, though if this data could have been captured at a herd-level it could have been compared to the BTM antibody results. This could have been a proxy to determine how much of an impact *M. bovis* was having within the herd.

3.5.7 Recruitment

Farmers were provided with information on the BTM study and were able to opt-in if they wished to participate. This method of recruitment can lead to self-

selection bias as farmers who may have a particular interested or experience in the subject area are more likely to enrol (Torrence, 1997). *M. bovis* has become an increasingly important pathogen to agriculture and veterinary industries worldwide and is often mentioned in news publications in the United Kingdom. Farmers who have recent or past experiences with *M. bovis* in their herd may have been more likely to participate in this study than those who have never heard of the pathogen. Over 80% of farmers were aware of *M. bovis* prior to participating in the BTM study, as shown in Chapter 4.

To ensure that participation in the BTM study was accessible to all dairy farmers, farmers could enrol via post, email, or phone. Farmers were also made aware of the study via an information flyer received by direct mail. Enrolling participants by direct mail is an efficient method of recruiting older participants (Bonk, 2010). With the majority of farmers in the UK aged 55 and older (Scottish Government, 2021) it was important to the researchers to ensure that enrolment and participation was available to as many dairy farmers as possible. Many farmers also chose to enrol via phone or email, which were quick, convenient methods for contacting the researchers.

In the second phase of BTM study recruitment, vet practices that were known to the researchers to have large numbers of dairy clients were approached and asked to encourage uptake by their clients. Almost 30 farms enrolled in the BTM study during the second round of recruitment via their registered veterinary practice. Despite promotion of the BTM study, these farmers may have been unaware of the study until they were contacted by their vet, or they may have previously not wished to participate for various reasons. This could have also increased the potential for selection bias as vets may have mainly approached their clients with recent or suspected *M. bovis* in their herd to participate, which could produce a higher prevalence estimate.

The longitudinal BTM study took place between August 2020 and October 2021 during a time that had a significant impact on the production of scientific research due to the ongoing COVID-19 pandemic (Raynaud et al., 2021). This likely to have had an effect on a few aspects of the BTM study recruitment and participation. The recruitment was put on hold for five months at the start of the pandemic, causing a loss of momentum, which led to a small number of

farmers dropping out prior to the commencement of sampling. Participant dropout, also known as attrition, is a common, and often unavoidable, occurrence in longitudinal studies (Jacobsen et al., 2021). Dairy farmers are notoriously busy with the day-to-day running of a farm therefore it was expected to be a challenge to remind farmers about sampling. Farmers may have dropped out of the study due to forgetting about the study or a perceived lack of time to collect the samples.

Individual results were sent to every farmer after each sampling point as an incentive to maintain their engagement. After receiving one or multiple results, some farmers may have felt that they had received sufficient information on the *M. bovis* status of their herd which resulted in them dropping out.

3.5.8 Limitations of questionnaires

As questionnaires are retrospective, they rely on study participants recalling information or having records with the information that is required (Dörnyei and Taguchi, 2009). In both the BTM study and calf study, the majority of questions could be answered by the farmer physically going to look at the farm set-up or recording how day-to-day tasks were carried out. In a few questions in both the BTM study (Q1, Q3 and Q16) and the calf study (Q1, Q8, Q9 and Q18), farmers would have likely looked at farm records or medicine books to answer correctly.

The potential for recall bias in Q14 of the BTM study cannot be ruled out. In this question farmers were asked if they were aware of *M. bovis* prior to participating in the study. Recall bias can occur when study participants are not able to accurately describe details resulting in information being incorrect or omitted (Talari and Goyal, 2020). The significance and impact of a particular event to an individual influences the accuracy of that memory (Smith et al., 2003). Farmers may not have known whether they were aware of *M. bovis* or not. Additionally, there could have been some confusion with *Mycobacterium bovis* (also abbreviated to *M. bovis*), which causes bovine tuberculosis, resulting in respiratory disease in youngstock (Verteramo Chiu et al., 2019).

Furthermore, the choice of words in a question can influence the responses of study participants. Awareness is defined in the Oxford Dictionary as the "knowledge or perception of a situation or fact", and the "concern about and

well-informed interest in a particular situation or development". With regards to Q14 in the BTM study, prior awareness of *M. bovis* could have been interpreted as hearing about *M. bovis* and having no knowledge of what it is, or a more thorough understanding of *M. bovis* (e.g. symptoms, transmission, treatment, or significance in the dairy industry). This question could have been phrased differently, for example, "Had you heard of *Mycoplasma bovis* before participating in this study?".

Only one question in the BTM study was hugely misinterpreted, or rather difficult for participants to answer, that was where they were asked how often they clean and disinfect calf feeding equipment. The apparent confusion when answering this question could have been avoided by creating one question asking about cleaning and the other disinfecting of feeding equipment. This issue also highlights the difficulty of trying to fit farmers' complex management into categories in questions with pre-defined choices.

Recall bias may also occur where farmers have reported a particular behaviour that does not match their actual behaviour, as they may have wanted to write what they felt was the 'correct' answer. Although the topics addressed in both studies were not invasive, the farmers may intend to do the best thing, but circumstances prohibit this.

3.5.9 Conclusions

Although the results presented in this chapter were produced from two studies on *M. bovis*, the findings have provided some insight into the general herd structures and management practices of dairy farmers in Scotland.

There is always the potential for selection bias in a study as a result of the recruitment approach. It is not known if the results are an accurate representation of current practices in Scotland, however the sample appeared to capture a representative and diverse range of farmers from across the country.

The reasons why farmers are choosing specific practices addressed in the studies is not known and thus can only be speculated. Future research could aim to understand the drivers behind Scottish farmer behaviours and decision making.

Chapter 4 Prevalence of *Mycoplasma bovis* in the bulk tank milk of Scottish dairy herds and associated risk factors

4.1 Introduction

M. bovis was first identified in the United States in the 1960s in a case of bovine mastitis (Hale et al., 1962).

M. bovis is now recognised as one of the pathogens involved in the multifactorial BRDC in youngstock (Kusiluka et al., 2000). It also causes a variety of other conditions including *Otitis media* in calves, mastitis and arthritis. Clinical signs can range from acute to chronic, although some cattle remain asymptomatic but with the ability to infect others (Houlihan et al., 2007).

Transmission occurs via direct contact with nasal secretions, semen (Haapala et al., 2018), milk and colostrum fed to calves (Stalheim and Page, 1975), *in utero* (Pfützner and Schimmel, 1985), and by aerosol (Maunsell et al., 2011). Additionally, *M. bovis* can survive in the environment for months on bedding (Justice-Allen et al., 2010), and may also contaminate feeding equipment (Schönecker et al., 2020), farmers' clothing, milkers' hands, and the milking parlour, which all serve as a potential source of infection. Several factors have been reported as risk factors for *M. bovis:* using a separate calving pen (Haapala et al., 2018), large herd size (Fox et al., 2003), purchase of cattle (Fujimoto et al., 2020), and stress-factors (Aebi et al., 2015).

The diagnosis of *M. bovis* previously relied on culture methods that were slow and technically demanding, but the advancement of molecular methods such as PCR testing has led to improved diagnostics. PCR tests have a very high sensitivity and can therefore detect *M. bovis* early when there are lower bacterial loads. This is one of the advantages of using PCR even though the bacterial contamination is diluted in the BTM, PCR can still detect very low quantities of the pathogen. Furthermore, as PCR tests are also highly sensitive, larger outbreaks can be prevented as these tests can detect *M. bovis* at the early stages of infection prior to the onset of clinical signs.

ELISA tests are also a useful tool to determine whether individuals have been recently exposed to the pathogen; commercial ELISAs are available to detect antibodies to *M. bovis* in milk and blood. An issue with testing the BTM is that all ELISA tests have been validated in blood samples from calves, and in some also in individual cows (Veldhuis et al., 2023). Therefore, the reported sensitivities and specificities may not hold true for testing BTM samples.

Testing BTM with PCR and ELISA tests can be complementary testing to within-herd testing of individual animals. It is a cheap and easy method of testing that provides a snapshot of what is going on in the herd. Repeated testing of the BTM can indicate whether further testing of individual animals is required. If *M. bovis* is detected in individual cows or calves, farmers can then target the antimicrobial treatment, thus reducing the risk of AMR. As described in Chapter 1, limited antimicrobials are effective at treating *M. bovis* due to the pathogen's unique structure and action. Consequently, there is an increased reliance on CIAs such as Macrolides Tetracyclines, Lincosamides and Fluoroquinolones to treat *M. bovis* (Maunsell et al., 2011). CIAs are reserved for specific human use (European Medicines Agency, 2020).

In the UK (as in other countries), policy is driving the movement towards reducing antimicrobial use in veterinary medicine, thus putting the onus on farmers to adopt preventive measures via herd health planning and increased vaccine uptake (Gov, 2018). This highlights the importance of increasing our understanding of this pathogen to identify more efficient methods of control.

Prior to conducting the current study, the prevalence of *M. bovis* in Scottish dairy cattle was unknown, therefore the aims of this study were to:

I) estimate the bulk milk prevalence of both a) *M. bovis* DNA, to identify active infection, and b) antibodies to *M. bovis,* to detect exposure in Scottish dairy herds, and

II) identify potential risk factors associated with the presence of *M. bovis* antibodies in the BTM

4.2 Materials and methods

4.2.1 Study population and recruitment

A longitudinal BTM study was carried out in Scottish dairy herds. The study design and recruitment are detailed in Chapter 2. Briefly, the study was advertised to dairy farmers in Scotland between February and October 2020 (with a 5-month hiatus during the COVID-19 pandemic). Farms were recruited to the study between August and December 2020. Participants were asked to send four BTM samples; one every four months for one year. The samples were tested by the Applied Biosystems VetMAX *M. bovis* PCR test kit for the presence of active *M. bovis* infections and the ID Screen® *Mycoplasma bovis* indirect ELISA kit (IDvet, Grabels, France) for the presence of *M. bovis* antibodies. A short questionnaire on herd management practices was issued to farmers during the first sampling point, with the responses used to formulate a risk factor analysis.

The target and source population was dairy farms located in Scotland, which in February 2020 was 880 (SDHA, 2020).The sample size calculation indicated that a minimum of 88 farms was required to estimate the prevalence in a population size of 880, assuming a perfect diagnostic test, with an expected prevalence of 50%, a desired level of confidence of 95% and precision of 10% (Sergeant, 2018a). Recruitment was via an ‘opt-in’ process, see Chapter 2 for details.

Ethical approval was obtained from the University of Glasgow School of Veterinary Medicine ethics committee.

4.2.2 Questionnaire design

Potential routes of transmission of *M. bovis* that could introduce or maintain the disease within a herd were considered for inclusion in the study questionnaire (Figure 4-1). A copy of the questionnaire is included in Appendix 2.

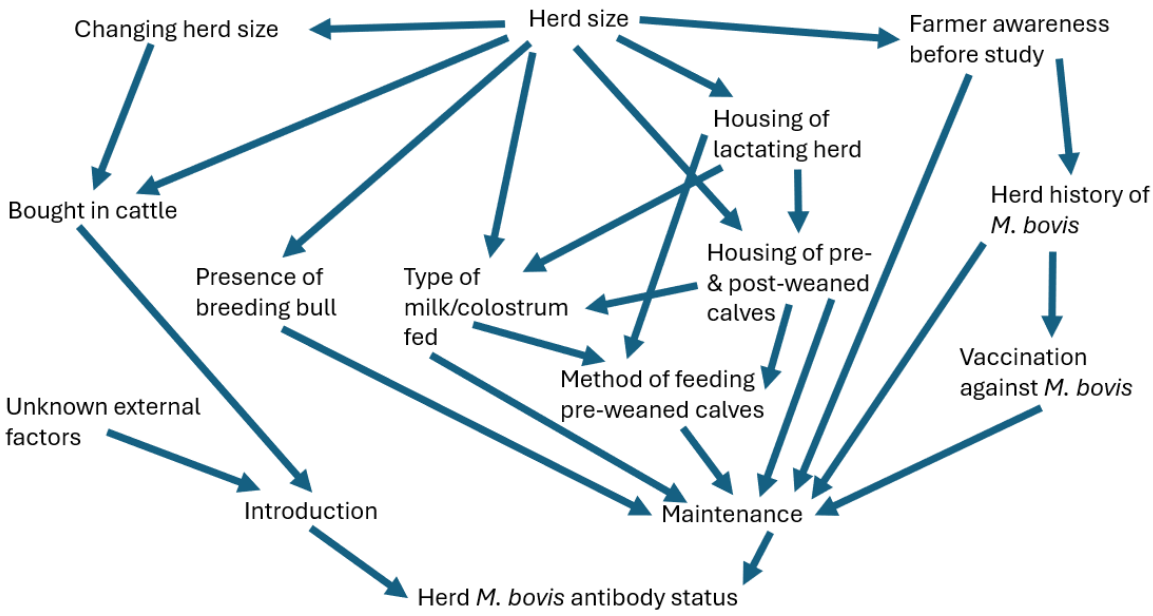


Figure 4-1: Causal web of potential factors associated with the introduction and maintenance of Mycoplasma bovis antibodies in BTM

In addition, a submission form was included to record: date of sample collection, the most recent bulk tank SCC, number of cows contributing to the tank at the time of sampling, number of cows not contributing to the tank due to illness or drug treatment at the time of sampling, and the number of dry cows. The submission form can be seen in Appendix 2.

4.2.3 Sampling

On receiving an expression of interest in participating from farmers, a study sampling kit was sent via post. Sampling kits contained a participant information sheet, consent form, sampling instructions, a short questionnaire on general herd management practices (Table 4-1, full questionnaire in Appendix 2), two sampling tubes each containing a preservative tablet, a sample submission form, and an envelope with pre-paid postage return.

Table 4-1: Summary of the questions asked to farmers to identify potential risk factors associated with the presence of *M. bovis* and antibodies to *M. bovis*

Subject	Description
Herd structure	Herd size (0-12 month old calves, 12-24 month olds, cows, breeding bulls), number of bought in cattle (0-12 month old calves, 12-24 month olds, cows, breeding bulls), changing herd size (increasing/maintaining/decreasing), rearing of dairy

Subject	Description
	bull and beef calves, length of time rearing dairy bull and beef calves
Calving	Calving period (block/year-round), block calving months (spring/summer/autumn/winter), use of separate calving pen
Housing	Lactating cow housing (permanent/semi-permanent/seasonal/maximum grazing), pre-weaned calf housing (individual/group), mixing of post-weaned calves (mixing/no mixing)
Milk and colostrum feeding	Type of colostrum (artificial/cows/both), type of milk (artificial/cows/both), pasteurisation of colostrum, pasteurisation of milk, feeding equipment (individual/group), cleaning of feeding equipment
Herd history of *M. bovis*	Farmer awareness of *M. bovis* prior to study, herd history of *M. bovis* (never present/never considered/suspect/confirmed with diagnosis), use of vaccine against *M. bovis*

Participants were asked to collect two bulk milk samples at the same time and return them via the pre-paid postage envelope along with the signed consent form, questionnaire and sample submission form.

4.2.4 Laboratory procedures

Bulk milk samples were frozen at -20°C upon arrival at the SRUC (Scotland's Rural College) Disease Surveillance Centre in Dumfries, then sent to SRUC Veterinary Services Veterinary and Analytical Laboratory, Edinburgh for testing. One of the two samples per farm was tested for the presence of *M. bovis* using the Applied Biosystems VetMAX *M. bovis* PCR kit following the manufacturers methods, and the other sample was tested using the commercially available IDvet *M. bovis* screen indirect ELISA for the presence of antibodies to *M. bovis*. The methods are described in Chapter 2.

The OD was calculated as followed, where the OD is expressed as the percentage positive (PP):

$$S/P\,\% = \frac{OD_{sample} - OD_{NC\ milk}}{OD_{PC\ milk} - OD_{NC\ milk}} \times 100$$

A PP value of ≥30% was considered positive as recommended by the manufacturer. The sensitivity and specificity are reported by the manufacturer

as 95.7% (95% confidence interval= 87.3 - 100.0%) and 100% (95% confidence interval= 96.3 - 100.0%), respectively. From field experience, the specificity is likely to be 98% (C. Mason, Personal Communication).

4.2.5 Statistical analyses

Representativeness of study population

To assess the representativeness of the study population, Wilcoxon rank sum tests were performed to compare the geographical distribution, and herd size of the study and the total dairy herd populations in Scotland.

Estimating prevalence at each sampling point

A case was defined as a herd with an ELISA-positive bulk milk sample based on the test cut-off of 30%. Apparent prevalence was defined as the number of farms with a positive BTM sample, divided by the total number of farms sampled. To correct for an imperfect test, the true prevalence was calculated using the test sensitivity and specificity of 95.7% and 100%, respectively, and using the Wilson confidence interval of 95% (Sergeant, 2018a).

Risk factor analysis

Data were cleaned and re-structured in Microsoft Excel before importing into RStudio (Posit team, 2022) for analysis. Explanatory variables generated from the questionnaire responses with less than five observations in a category were grouped where possible for the analysis. If combining of the groups was not possible, the responses were only reported in Chapter 3 and not taken forward to the univariable or multivariable analysis.

A risk factor analysis was performed on the ELISA test results of the BTM sample from the first sampling point. BTM results were analysed as a binary result, either positive or negative, according to the recommended cut off). All possible explanatory variables were individually tested by univariable logistic regression analysis and chi-squared testing with the binary BTM result as the outcome. Multivariable analysis was carried out using logistic regression.

To check for multicollinearity between explanatory variables that met the criteria for inclusion in the multivariable analysis ($p < 0.2$), the VIF was calculated. Any variables with collinearity were removed from the model.

A backwards stepwise selection process was applied to construct the multivariable models. The variable with the highest p-value was removed and p-values for the remaining variables were recalculated. Variables were removed one by one until the best fit model was identified. The 95% Confidence Intervals (CIs) and p-values were computed using a Wald z-distribution approximation.

Alternative selection criteria for explanatory variables were applied to generate other models: including all explanatory variables in the multivariable model and selecting variables that the author believed were more likely to be associated. This was to ensure that the chosen method created the best model for the data. A modification of the AIC known as the AICc was computed using the package *AICcmodavg* (Brewer et al., 2016). This is used instead of the AIC for smaller sample sizes. The AICcs were used to compare the models and to enable selection of the best model containing the least number of covariates that explained most of the data (Brewer et al., 2016). The model with the lowest AICc was accepted as providing the best fit to the data.

4.3 Results

4.3.1 Recruitment of farms

Farmers from 192 dairy herds in Scotland expressed their interest in participating in the present study, and 181 (94%) were ultimately enrolled.

Of the eleven dairy herds that did not continue in the study, seven expressed interest in the first round of recruitment prior to the COVID-19 pandemic. Two of these farms were unable to continue when sampling finally began as the herds had been sold. The four of the 11 herds that expressed interest in the second round of recruitment did not return any samples.

Samples were received from three unknown farms with no farm name. Despite contacting every farmer who expressed interest, the authors were unable to match these samples to a farm and consequently these samples were not included in the study.

4.3.2 Representativeness of study population

The 181 farms enrolled in the study made up 21% of the total dairy herd population in Scotland (n=879) at that time (SDHA, 2021). The geographical distribution of participating farms and the total dairy population in Scotland is shown in Figure 4-2 and Table 4-2. The largest proportion of dairy farms were located in the South and West of the country, Dumfries and Galloway (n=81), followed by Ayrshire (n=40) and Lanarkshire (n=16).

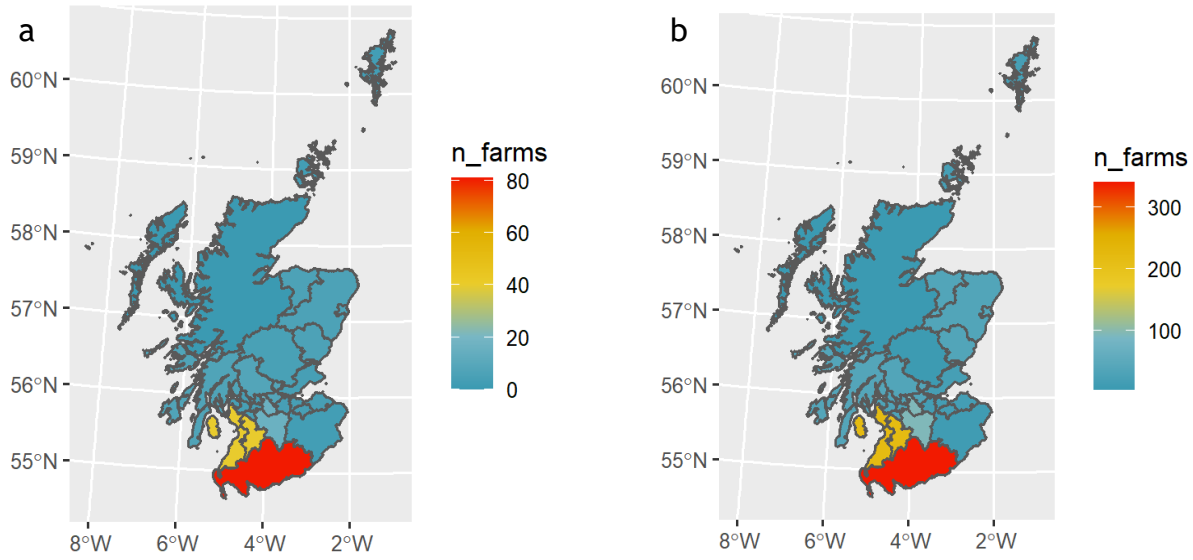


Figure 4-2: Choropleth maps illustrating the distribution of participating farms (a) and of total dairy herd population (b) in Scotland

Wilcoxon rank sum testing determined that there was no difference between the proportional distribution of study farms and of all dairy herds in Scotland (P>0.05).

Table 4-2: Percentage of study herds and the total dairy herd population by region in Scotland

Regions	Participants (%)	Total population in Scotland (%)
Aberdeenshire, Angus & Moray	3	4
Ayrshire	22	25
Argyllshire	4	4
Stirlingshire & Clackmannanshire	4	4
Perth & Kinross	3	1
Fife	2	2
Dumfries & Galloway	45	39

Regions	Participants (%)	Total population in Scotland (%)
Highlands, Orkney & Shetland	2	2
Dunbartonshire & Renfrewshire	2	4
Lanarkshire	9	11
Lothian	2	2
Scottish Borders	2	1
Total	100	100

The mean herd size of the study population, based on the number of cows, was 245, and the mean herd size of the total dairy farm population in Scotland was 209 (SDHA, 2021).

Wilcoxon rank sum test determined that there was no difference in the herd size distribution of the study and total populations (Figure 4-3).

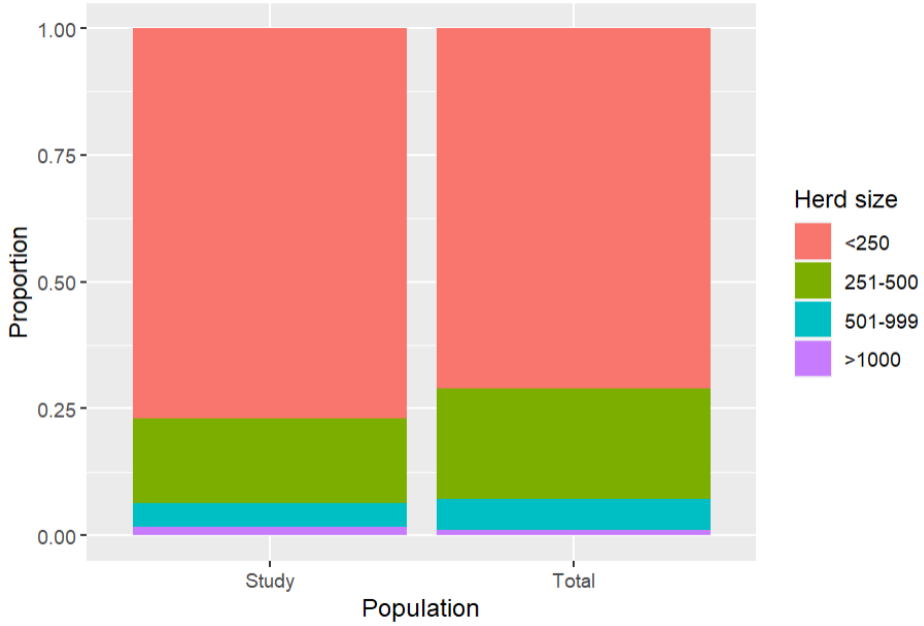


Figure 4-3: Distribution of total dairy herds in Scotland and study herds by herd size

4.3.3 Participants & samples

The timeframes of each sampling points are shown in Table 4-3.

Table 4-3: Timeframe for each sampling point

Sampling point	Timeframe
1	August to December 2020
2	January to April 2021
3	March to October 2021
4	July to October 2021

From the 181 herds that participated in the longitudinal study, a total of 578 BTM samples were received. The number of herds that contributed to each sampling point is shown in Table 4-4.

Table 4-4: Total number (and percentage) of herds that contributed to each sampling point

Sampling point	Number of herds (% of total)
1	181
2	154 (85)
3	135 (75)
4	108 (60)

Fifty-nine percent of herds that started the study completed all four sampling points. Thirteen percent of participants dropped out after the first sampling point (Table 4-5).

Table 4-5: Number of herds that sent in one to four samples

Number of samples/herd	Number of herds
1	23
2	25
3	27
4	106

Information on participation (including the geographical distribution of herds), the rate of dropout, and the time between samples is covered in detail in Chapter 3.

4.3.4 Prevalence estimates

ELISA results

Out of the 578 BTM samples received, 443 (77%) tested positive. The number of positive and negative samples for each sample period is shown in Figure 4-4. Seventy-six percent of herds tested positive in sample one (95% CI: 0.69-0.82), 71% (95% CI: 0.63-0.77) in sample two, 83% (95% CI: 0.76-0.88) in sample three, and 79% (95% CI: 0.70-0.85) in sample four. Overall, 86% (95% CI: 0.80-0.91) (n=156) of farms tested positive in at least one of their four samples.

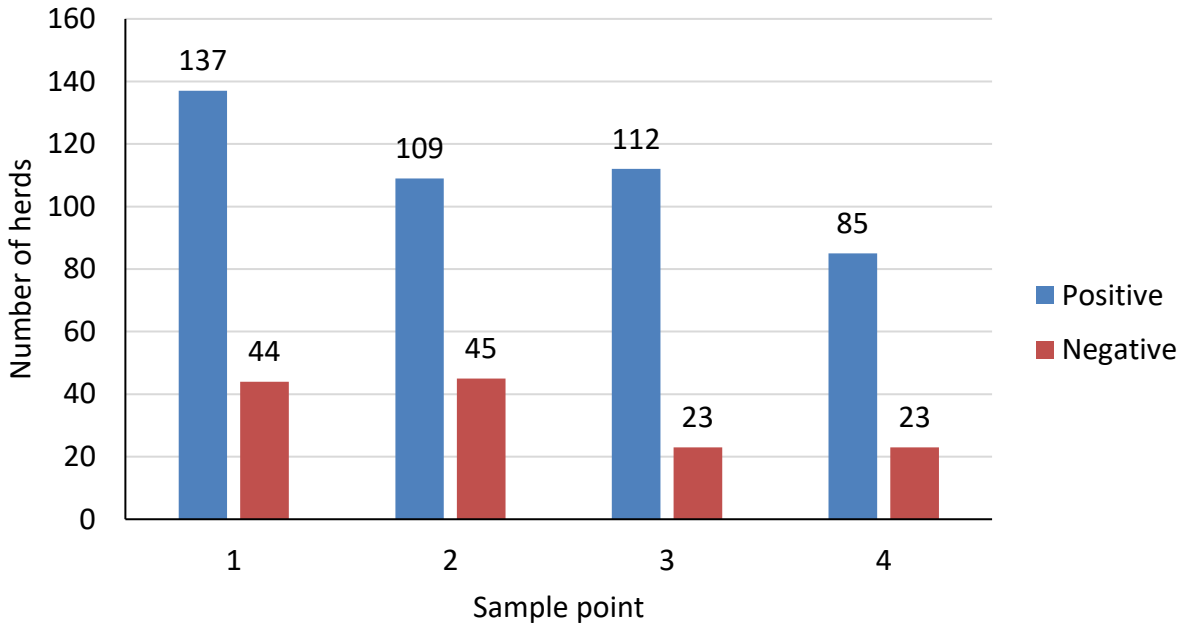


Figure 4-4: Number of positive and negative herds at each sampling point

The true BTM prevalence was estimated as 79% (95% CI: 0.72-0.85), 74% (95% CI: 0.66-0.81), 87% (95% CI: 0.79-0.93), and 82% (95% CI: 0.73-0.90) for samples 1 to 4, respectively, using the sensitivity and specificity of the manufacturer (sensitivity: 95.7% and specificity: 100%).

Seven herds recorded the use of a vaccine against *M. bovis*, two of which were autogenous vaccines. All seven herds tested antibody positive as expected.

PCR results

Only four herds tested positive by PCR for the presence of *M. bovis* DNA in the entire duration of the study. The number of PCR positive samples and sampling point in those herds are shown in Table 4-6.

Table 4-6: Results of the PCR positive herds

Farm	Number of positive samples	Sample point
A	1	2
B	2	1, 2
C	1	2
D	1	3

Three tested positive for only one sample and one herd tested positive for the first two consecutive samples. The herd-level prevalence was estimated as 0.01% (95% CI 0.001-0.031), 0.02% (95% CI 0.007-0.056), 0.007% (95% CI 0.001-0.041), and 0% (95% CI 0.000-0.034) for sampling periods 1-4, respectively (Sergeant, 2018b). In total five samples (1%) tested PCR positive out of the 578 samples received.

The PCR results were not taken forward for further statistical analysis, i.e. risk factor analysis, due to the small number of positive herds.

4.4.4 Factors associated with the BTM results

Univariable screening identified six variables that had a $p < 0.2$ (Table 4-7). The risk factor analysis was run with and without the seven herds that reportedly vaccinated against *M. bovis*, and the results were the same.

Table 4-7: Explanatory variables identified from the univariable screening with p < 0.2 that were taken forward to the multivariable regression

Variable	Category	OR	95% CI	Negative	Positive	P
Number of 12-24 month old calves	< 100	1		29	67	0.071
	> 100	1.95	0.96-4.11	14	63	
	Yes	1		36	123	0.165

Variable	Category	OR	95% CI	Negative	Positive	P
Rear dairy bull/beef calves	No	0.51	0.20-1.37	8	14	
Bought-in any cattle	Yes	1		21	84	
	No	0.58	0.29-1.14	23	53	0.114
Housing of lactating herd	Some or all permanently housed	1		7	46	
	Seasonal, semi-permanent housing or max grazing	0.37	0.14-0.86	37	91	0.029
Awareness of *M. bovis*	Not aware	1		12	21	
	Aware	2.14	0.93-4.78	31	116	0.067
Herd history of *M. bovis*	Never present	1		34	62	
	Suspected or diagnosed	4.06	1.92-9.27	10	74	0.0004

No variables obtained from the sample submission form were associated with the BTM result.

After calculating the VIF, there was little collinearity between the explanatory variables that were entered into the multivariable model, with the exception of 'Awareness' and 'Herd history'. Consequently, the variable 'Awareness' was not included in the multivariable analysis.

The final model generated from the multivariable analysis is shown in Table 4-8. The model contained only one explanatory variable, 'Herd History'.

The model's explanatory power was weak (Tjur's R2 = 0.07). The model's intercept, corresponding to herd history 'Never present', was at 0.60 (95% CI: 0.19, 1.03, p=0.005). The McFadden's pseudo R2 was 0.07 indicating that the model had no predictive power. Within this model, the effect of herd history

'Suspected to be present or diagnosed' was statistically significant and positive (beta = 1.40, 95% CI: 0.65, 2.23, p<0.001; Std. beta = 1.40, 95% CI: 0.65, 2.23).

Table 4-8: Results from the final multivariable model on the association between management practices and bulk tank milk positivity of *M. bovis*

Parameter	Categories	Odds ratio	95% CI		P
			Lower	Upper	
Herd history of *M. bovis*	Never present	Reference			
	Suspected to be present or diagnosed	4.06	1.92	9.27	0.0004

The results of comparing the final model to the two alternative models computing the AICc is shown in Appendix 6. The final model had the lowest AICc (190.19). The difference in AICc of Model B and Model C were both greater than two units compared to the final model, which is classed as a large difference (Arnold, 2010). The final model had the highest proportion of the total amount of predictive power of the dataset.

The two alternative models are shown below. Cows that were housed seasonally, semi-permanently or were grazed were less likely to be antibody positive in their BTM compared to herds with some or all cows housed permanently (Table 4-9 and Table 4-10). Also, herds that did not rear dairy bull and beef calves were less likely to test antibody positive in their BTM than herds that do rear dairy bull and beef calves (Table 4-9).

Table 4-9 - Alternative Model B from the multivariable analysis on the association between management practices and BTM seropositivity of *M. bovis*

Parameter	Categories	Odds ratio	95% CI	Negative	Positive	P
Cow housing	Some or all permanently housed	1		7	46	
	Seasonal, semi-	0.33	0.12 – 0.78	37	91	0.017

Parameter	Categories	Odds ratio	95% CI	Negative	Positive	P
	permanent housing or max grazing					
Rear dairy bull/beef calves	Yes	1		36	123	
	No	0.41	0.35 – 1.14	8	14	0.078

Table 4-10 - Alternative Model C from the multivariable analysis on the association between management practices and BTM seropositivity of *M. bovis*

Parameter	Categories	Odds ratio	95% CI	Negative	Positive	P
Cow housing	Some or all permanently housed	1		7	46	
	Seasonal, semi-permanent housing or max grazing	0.37	0.14 – 0.86	37	91	0.029

4.5 Discussion

4.5.1 Prevalence estimates

To the author's knowledge, this is the first study to estimate the prevalence of *M. bovis* in bulk milk in Scotland.

Prevalence by ELISA

The true BTM prevalence estimations for the four sampling points ranged from 74% to 87%. These estimates are high compared to previous longitudinal BTM studies on *M. bovis* in dairy herds, which ranged between 0-60% (Table 4-11).

There have been very few studies to date estimating the BTM prevalence of *M. bovis* antibodies at herd-level in dairy herds and more studies reporting the

prevalence of *M. bovis* using culture or PCR. There are various reasons why the prevalence estimates in this study were higher than previously reported; the study design, diagnostic test(s) used, the dilution effect, and the duration of antibody production.

Cross-sectional vs longitudinal

The study design and methods used varied to different degrees across all of the prevalence studies listed in Table 4-11. All but one of the studies were cross-sectional studies sampling from the BTM at only one time point, whereas McCarthy et al. (2021) conducted a longitudinal study with three sampling points.

Although cross-sectional studies are a quick, easy and inexpensive method of capturing the current disease situation, a limitation is that the results may not be generalisable to the true disease status of each herd. BTM antibody levels can be very dynamic due to the constant, and sometimes unpredictable, changes in the cows contributing to the BTM (depending on factors such as the calving pattern, illness and treatment). Consequently, samples collected at different timepoints could produce different test results. Longitudinal studies are more likely to produce a truer picture of the prevalence of a disease such as *M. bovis* in the BTM, as they are not reliant on only one result. Therefore, the results of this study and that of McCarthy et al. (2021) are more likely to be true, compared to the cross-sectional studies.

Table 4-11: Herd-level prevalence estimations in previous studies of *M. bovis* antibodies and *M. bovis* in BTM samples, by culture methods, ELISA and PCR tests

Author(s)	Country/region of study	Sample(s) collected	Diagnostic test	Prevalence estimate
Prevalence of *M. bovis* antibodies by ELISA tests				
Gille et al. (2018)	Belgium	BTM	BIO K302, Bio-X Diagnostics S.A.	24.8% (95% CI 16.4-33.2%)
Hurri et al. (2022)	Sweden	BTM	ID Screen® *Mycoplasma bovis* indirect ELISA kit (IDvet, Grabels, France)	3.8% (95% CI 3.0-4.7%) (147/3,144)

Author(s)	Country/region of study	Sample(s) collected	Diagnostic test	Prevalence estimate
McCarthy et al. (2021)	Ireland	BTM	BIO K302, Bio-X Diagnostics S.A	53% (95% CI 39.5-68.4%)
			ID Screen® *Mycoplasma bovis* indirect ELISA kit (IDvet, Grabels, France)	0.42% (0)
				30% (95% CI 21-41%)
McAloon et al. (2022)	Ireland	BTM	ID Screen® *Mycoplasma bovis* indirect ELISA kit (IDvet, Grabels, France)	45% (95% CI: 42–47%)
Penterman et al. (2022)	Netherlands	BTM	BIO K260, Bio-X, Diagnostics, Rochefort, Belgium	60% (12/20 farms)
Mõtus et al. (2021)	Estonia	BTM	Monoscreen Ab ELISA (Bio-X Diagnostics S.A.)	20.0% (24/120, 95% CI 13.1-28.3)
McAloon et al. (2024)	Ireland	BTM	BIO K302 ELISA, Bio-X Diagnostics S.A And ID Screen® *Mycoplasma bovis* indirect ELISA kit (IDvet, Grabels, France)	12% (88/728) And 56% (406/728)
Prevalence of *M. bovis* pathogen by PCR and/or culture methods				

Author(s)	Country/region of study	Sample(s) collected	Diagnostic test	Prevalence estimate
Hurri et al. (2022)	Sweden	BTM	PCR (PathoProof Mastitis Major 4, Thermo Fisher Scientific)	0% (0/3,144)
Ninković et al. (2024)	Serbia		PCR	9.57% 11/115 (95% CI 4.87-16.47)
Gille et al. (2018)	Belgium	BTM	PCR (PathoProof Mastitis Complete 16 PCR assay)	7.1% (95% CI 2.1-11.5%)
McCarthy et al. (2021)	Ireland	BTM	PCR developed by Sachse et al. (2010)	0%, 1%, and 0%
Bauman et al. (2018)	Canada		PCR (PathoProof Mastitis Major 4, Thermo Fisher Scientific)	0% (2/370)
Olde Riekerink et al. (2006)	Prince Edward Island	BTM	Culture	2.1% (4/193)
Murai and Higuchi (2019)	Japan	Milk and BTM	PCR Cica geneus Mycoplasma bovis Detection Plus Kit (Kanto Chemical Co. Inc., Tokyo, Japan)	3.8% (30/784)

Author(s)	Country/region of study	Sample(s) collected	Diagnostic test	Prevalence estimate
Wen, Zhang and Hao (2019)	China	Milk & serum	Culture (PPLO agar), PCR, ELISA (Biovet, Saint-Hyacinthe, Canada)	53% (out of 850)
	Inner Mongolia Autonomous region			34.2% (out of 860)
Passchyn et al. (2012)	Belgium	BTM	Culture - modified Hayflicks media And PCR - tDNA intergenic spacer (Stakenborg et al., 2005)	1.5% (3/200) from one of the three samples in herds
Pinho et al. (2013)	Portugal	BTM	QIAamp Blood Kit (Qiagen, Hilden, Germany)	2.4% (4/164)
Gioia et al. (2021)	USA	BTM	As described by Gioia et al. (2016)	75.1% of 855 isolates from 98 herds
McAloon et al. (2024)	Ireland	BTM	PCR developed by Sachse et al. (2010)	1.0% (7/728)

The recruitment strategy can also influence the prevalence estimates. Where it has previously been speculated that the low BTM prevalence estimates could be attributed to the fact that some farmers may fear judgement and avoid participation in disease surveillance studies (Bauman et al., 2018), it could be argued that the opposite has occurred in the present study. The recruitment of farmers to this study relied heavily on farmers choosing to participate, particularly in the latter stages of recruitment where vets encouraged their clients to register. At the time of conducting this study, *M. bovis* was a widely

discussed disease in the UK veterinary industry and it has had lots of media attention in recent years. Therefore, farmers who were experiencing problems with *M. bovis* (or thought that they might be) may have been more inclined to participate. This could have led to a higher estimation of prevalence. Likewise, during the second round of recruitment, vets who were encountering this disease more frequently in practice may have targeted specific clients who had recent issues with *M. bovis*, or issues that could have been due to *M. bovis*, to participate rather than herds with no history of the disease. The recruitment approach used in this study introduces a potential for selection bias as vets may have selected clients with a known history (or one that was indicative) of *M. bovis* which would again lead to a higher estimated prevalence. A similar approach was used by McCarthy et al. (2021), who promoted their study to Irish dairy farmers via stakeholders and media campaigns.

On the other hand, Gille et al. (2018) applied a random selection stratified by province to select farms, and both Hurri et al. (2022) and McAloon et al. (2022) randomly selected BTM samples and herds from laboratories performing routine quality testing of BTM. Random selection was not used in the present study as it was not possible to obtain contact information or any personal details of dairy farmers throughout Scotland due to data sharing issues. During the initial planning stages of this study, one option was to approach national milk recording companies to recruit farms. It would be possible to receive BTM samples via this method, however, the milk recording companies would not share any information about the farm. Consequently, questionnaires could not be issued to the farmers as there would be no communication between the author and the farmers. Furthermore, repeated samples could not be taken due to data protection.

Ultimately, this method of sampling would impede the author's ability to answer two research questions in this thesis: a) how the BTM prevalence changes over time, and b) identifying management practices or herd structures that were associated with the BTM prevalence. Aside from the impact this would have on the study design, it would have changed a key feature of this study, which was the communication between the author and farmers.

While the potential for selection bias in the present study is possible, the high BTM antibody prevalences reported in this study could simply be due to *M. bovis* being more prevalent in Scotland compared to other countries.

Tests used and sampling approach

Another factor that may influence the prevalence estimates is the diagnostic tests used. Most of the previous studies estimating the BTM prevalence of *M. bovis* antibodies used the ID screen indirect ELISA, likely as it is a cost-effective and convenient commercially available test. According to the manufacturer, the IDvet ELISA test has a high reported sensitivity and an even higher specificity (100%), therefore there should have been no false positive herds in the study. Although, the ELISA test specificity may be lower, as reported by various authors, at animal level; 98.6% (Andersson et al., 2019), 97.7% and 99.9% (Marquetoux et al., 2023), and 99.3% (Veldhuis et al., 2023). Consequently, with a lower specificity there is an increased chance of false positives, and therefore a lower true prevalence than has been reported in this Scottish study. The sensitivity of the ID screen test was also reportedly lower from the same studies; 93.5% (Andersson et al., 2019), 72.8% and 66.0% (Marquetoux et al., 2023), and 92.5% (Veldhuis et al., 2023), meaning that there may be a higher number of false negatives.

Other studies listed in Table 4-11 used ELISAs developed by Bio-X, the Bio K302 and Bio K260, both of which have been compared to the performance of the ID screen ELISA test. Both the Bio K260 and the Bio K302 have reportedly lower sensitivities compared to the ID screen ELISA when used on samples from individual animals, 14.1% (Veldhuis et al., 2023) and 49.1% (Andersson et al., 2019), respectively. Likewise, the specificity was also lower than the ID screen ELISA, 97.2% for the Bio K260 (Veldhuis et al., 2023) and 89.6% for the Bio K302 (Andersson et al., 2019). The IDvet ELISA clearly has the highest sensitivity and specificity, therefore enables the best opportunity to detect true positive and negative animals.

A more recent study compared the sensitivity and specificity of the ID screen ELISA to the Bio-X Bio K302 ELISA in BTM samples in Ireland (McAloon et al., 2024). The performance of the tests was compared in various models. The sensitivity of the ID screen and Bio K302 ELISAs was 94% and 22%, respectively,

and the specificity was 92% and 97%, respectively. In another model in which the population was stratified by region, the sensitivity of the ID screen ELISA was slightly higher (95%) whereas the specificity was lower (88%). In the same model, both the sensitivity and specificity of the Bio K302 increased slightly, 24% and 98%, respectively. By applying those sensitivity and specificity estimates to the present study, the true prevalence estimates would not change drastically. For example, for the first BTM samples, the true prevalence would be 79% (95% CI: 0.71-0.86) using the sensitivity and specificity in the first model and 77% (95% CI: 0.68-0.84) using the sensitivity and specificity of the second model. Both of these estimates are slightly higher than the estimate using the sensitivity and specificity reported by the manufacturer.

Diagnostic tests are often validated on a specific group of animals, such as calves. Therefore, the test cut-off for a positive result may not be reliable for different age groups which could alter the sensitivity and specificity. Petersen et al. (2020) suggested that the sensitivity of the ELISA test may be dependent on the level of antibodies. In summary, older cows testing positive may have been infected for a long period of time, therefore it is more likely that historic infections are detected. Conversely in younger animals, the detection of antibodies may be more indicative of recent infection. In a more recent study, Veldhuis et al. (2023) reported a high sensitivity of the ID screen ELISA (92.5%) and observed no differences in the sensitivity between samples from cows, calves or herds that had had an outbreak of clinical disease. This finding is interesting because diagnostic tests often show variability in the sensitivity and specificity across different age groups of animals. For example, immune responses can differ, with adult cows having more developed immune systems compared to calves, which can lead to less detectable antibodies in younger animals. However, according to the findings of that study, this did not appear to affect the ELISA's performance. Moreover, the lack of difference in sensitivity suggests that the negative predictive value (NPV) of the test should remain consistent when testing cows or calves, indicating that there will be a reliably high proportion of true negatives regardless of the age of the animals tested.

Furthermore, variability in test performance can occur when analysing different types of samples, for example, blood and milk. According to the manufacturer of the ID screen ELISA test, there was excellent agreement in the results of testing

milk and blood (kappa correlation coefficient = 0.86). This suggests that there should not be a significant difference in the sensitivity and specificity of the ID screen ELISA test when used on calves or cows.

Although there appears to be no difference in the test performance when sampling from cows or calves, there will likely be a difference in the sensitivity when testing BTM samples. This will mainly be due to the dilution of antibodies as the BTM contains milk from all cows rather than just one. If only a small proportion of cows are producing antibodies, there may not be enough detectable antibodies above the test cut-off. Consequently, the ELISA may have a lower sensitivity and thus there could be more false negatives. The dilution effect of BTM samples should not have an impact on the specificity. The specificity is the ability of the test to correctly identify healthy individuals/samples. A higher specificity reduces the number of false positives. The dilution of antibodies in the BTM sample will not increase the likelihood of detecting false positives.

Cows contributing to the BTM and the duration of antibody production

One factor to consider in relation to the prevalence estimates is the lack of knowledge about the relationship between *M. bovis* antibody levels in individual cows and in the BTM. It is not known what proportion of positive cows, nor the volume of antibodies that they need to shed in order to obtain an antibody positive BTM result. Vähänikkilä et al. (2019) reported an association between the mean antibody concentration in serum from individual cows and the antibody concentration in BTM sampled at the same time. This would be expected as the more cattle producing high volumes of antibodies, the higher the pooled BTM sample should be.

Furthermore, as discussed in Chapter 1, there is little data available on the length of time that antibodies are shed in milk, with only a few studies reporting the duration of shedding. Antibodies may be detectable for many months post-infection (Byrne et al., 2000; Vähänikkilä et al., 2019) but there are several factors that may influence the duration of antibodies in individual animals such as, the age of the animals, clinical manifestation, the pathogen load, vaccination and stress.

As younger animals have less developed immune systems, their antibody levels can fluctuate more, and it is likely that a positive antibody test is indicative of recent infection (Petersen et al., 2020). On the other hand, older cattle have stronger immune systems, therefore their antibody levels may remain more stable, but last for longer post-infection.

Also, the stage and type of infection can affect the detection of antibodies. The production of antibodies may peak at different stages of the infection and may vary with different levels of infection (e.g. subclinical, chronic and acute). Furthermore, if animals are continuously exposed or reinfected with *M. bovis*, then the level of antibodies detected may remain high for prolonged periods of time.

The immune system may be weakened by exposure to stressors such as overcrowded housing, transport and co-infections with other pathogens. Stress can prolong recovery from infections which in turn increases the duration of antibody production.

In the present study, it was not known how long it had been since an antibody BTM positive herd experienced a challenge with *M. bovis*. However, collecting repeated BTM samples over the study period provided valuable insights into the dynamics of antibody shedding. If the BTM samples from a specific farm continuously tested positive, then it was safe to assume that *M. bovis* was currently, or very recently, present within the herd. Likewise, if the BTM samples from a herd were continuously negative, this would suggest that *M. bovis* was not present or was present at very low levels. Regardless of the results, it cannot be known how much or little disease is present in the herd without testing individual animals. Research into the associations between individual cow milk OD% and BTM OD% should be prioritized, as a major question arising from an antibody-positive BTM sample is, 'What does this result signify?" In Chapter 5, the BTM OD% trends and the underlying reason for observing the trends are explored.

Prevalence by PCR

Only five BTM samples from four farms tested positive by PCR for the presence of *M. bovis* DNA in the present study, suggesting that there were extremely low

levels of active excretion of *M. bovis* in the study herds. This finding is consistent with the previous studies listed in Table 4-11, as most reported very low BTM prevalences. For example, McCarthy et al. (2021) reported only one PCR positive BTM sample out of the 120 Irish farms enrolled in the study. Similarly, only two herds (0.5%, out of 375) were positive by *M. bovis*-PCR in Canada (Bauman et al., 2018), and a prevalence of 0.56% was reported in Japan (Higuchi et al., 2011).

The LSI VetMAX™ *Mycoplasma bovis* kit used in this study has a high sensitivity and specificity and has been used previously in only a handful of studies. This PCR detected *M. bovis* in a small number of bovine semen samples from bulls in USA breeding centres (Yatsentyuk, 2022). Also, Oucheriah et al. (2022) used the same PCR kit to detect *M. bovis* respiratory disease in Algerian veal calves. Both studies reported a high sensitivity of the VetMAX™ PCR kit. To the best of the author's knowledge, this PCR kit has not been used to detect *M. bovis* DNA in BTM, likely as there are a range of different PCRs available.

The collection of BTM for testing is a quick and convenient method for assessing herd-level infection status, though there are a number of limitations to using PCR testing for the detection of *M. bovis* DNA in BTM. These limitations may explain the apparently low PCR prevalence estimates in this study and previous studies. Samples collected from the BTM contain only a subset of the herd, for example dry cows will not be lactating and thus will not contribute to the BTM. As discussed in Chapter 3, the proportion of dry cows at the time of sampling will be largely influenced by the calving pattern and time of year. Most importantly, infected cows will not be contributing to the tank if they are presenting clinical symptoms (Hazelton et al., 2020c). Likewise, if they are receiving antimicrobial treatment for clinical mastitis then they should also not be contributing to the tank. Consequently, positive animals may be missed from the herd, thus not providing a true picture of the herd-level prevalence. Although, not all cattle that are infected with *M. bovis* display any clinical symptoms (Punyapornwithaya et al., 2011), therefore testing BTM samples by PCR could offer the potential to identify herds with a high number of asymptomatic animals.

Similar to the ELISA test, a limitation with using PCR tests on BTM samples is that the sensitivity will be reduced, compared to testing milk from an individual cow. A positive result will only be obtained if enough actively infected cows that are shedding the pathogen are contributing to the BTM at the time of sample collection. As the BTM is a pool of milk from multiple cows, due to the dilution effect, a larger proportion of actively infected cows would need to contribute to a BTM sample, otherwise the presence of *M. bovis* DNA may not be detected. However, this PCR has a cycle threshold of 45, enabling the detection of very low quantities of *M. bovis* DNA.

Another factor influencing the sensitivity and specificity is the potential for intermittent shedding of infected individuals wherein the shedding of the pathogen from infected individuals is inconsistent (Byrne et al., 2005). Shedding of infected individuals within the herd will likely not be synchronised, therefore positive individuals could be diluted in the bulk tank making the overall result negative when in fact there is a proportion of actively infected cows.

A limitation with using PCR tests is that they cannot distinguish between viable and non-viable organisms, therefore it is not known whether a PCR positive sample reflects an active or a historic infection.

Aside from the limitations of using PCR to test BTM samples, the very low reported prevalences across various studies raises a question as to whether cows' milk is a significant risk factor for *M. bovis* spread within a herd. Furthermore, a recent study reported a very low prevalence of *M. bovis* in colostrum samples in Scotland (1.3%) (Denholm et al., 2024). Although *M. bovis* has been detected in milk and colostrum from infected cows, it may not transmit as readily to youngstock via this route. Further research is required to better understand the most important routes of transmission within farms.

If a herd is consistently PCR negative over subsequent BTM samples, it could be assumed that the herd does not have the disease. Though it cannot be known for sure without testing individual animals, both within the lactating herd and youngstock. It is possible that *M. bovis* could be present in other groups of cattle on the farm, but due to good biocontainment, the disease doesn't spread to the lactating herd. This topic is discussed further in Chapter 6.

PCR testing of BTM samples should not solely be relied upon for detecting *M. bovis* at herd-level, and it is likely better to also use ELISA tests as antibodies are more detectable in the BTM than *M. bovis* DNA.

Summary of BTM prevalence estimates

Although the antibody-prevalence in Scottish dairy herds is apparently high, the question remains whether this translates to high incidence of clinical mastitis within-farm. In other words, is *M. bovis* significantly affecting production and animal welfare in these positive herds or are a large number of infected cows asymptomatic? In the present study, associations were made with positivity but not with clinical disease. Further studies are needed to explore the production and economic impact of *M. bovis* within Scottish dairy herds. Another question this study raises is how many positive cows have contributed to the BTM samples that were positive. There are no studies on the relationship between the number of antibody-positive lactating cows and bulk milk status specifically, though Petersen et al. (2016) reported that herds with higher prevalence of antibody-positive cows have increased bulk milk ELISA optical density measures.

Only 24% of herds in the present study were consistently antibody-negative, suggesting that these herds had not been exposed to *M. bovis* or that the number of cows within the herd that were recently infected was low. It still would not go a miss to test individual animals across different groups as there is the possibility that due to good biocontainment the disease has not spread to all groups within the herd. Furthermore, negative herds are clearly less common, and it is recommended that they should be protected via biosecurity practices to prevent the introduction of *M. bovis*.

The number of antibody-positive herds was considerably higher than that of PCR positive herds. The majority of antibody-positive herds in the present study likely contained a proportion of actively infected cows around the time of sampling. As discussed previously, detecting active infection in BTM can be challenging for a number of reasons: the intermittent shedding patterns and symptomatic cows removed from the BTM for treatment. If there were any PCR positive cows contributing to the BTM at the time of sampling, they could have remained undetected as the infected milk would have been diluted in the bulk tank. Furthermore, antibodies are thought to be present for months following

infection with *M. bovis* which could explain the differences between the high ELISA prevalence and low PCR prevalence.

Ultimately, *M. bovis* may be more prevalent in Scotland than in other countries due to various reasons not known at present. The high BTM antibody prevalence in the present study would support this.

4.5.2 Herd history of M. bovis

Herds with suspected *M. bovis* presence were more likely to be antibody positive than herds that the farmer had either never considered or did not believe *M. bovis* was present, which suggests that farmers should trust their instinct and if they suspect that *M. bovis* may be present, then it likely is.

A limitation with the factor 'herd history' is that originally, the question was split into five multiple choice options. When comparing the answers to the BTM results, the sample sizes were too small, therefore the answers were grouped. It would have been beneficial to be able to compare the BTM antibody results of farms in which *M. bovis* was not believed to be present, to those that suspected and where it had been diagnosed, separately.

Farmers were not asked to quantify 'recent' *M. bovis* presence (or suspected presence), so for some, recent could have been one month prior, and for others this may have been six months prior. This information could have been beneficial to collect as it may have indicated how long antibodies are detectable in the BTM post-infection. Antibodies can be shed for many months following an infection with *M. bovis* (Ruhnke et al., 1976), therefore participating herds where *M. bovis* was reportedly diagnosed recently, were likely to test antibody positive.

Unlike the other risk factors identified in the study, the herd history of *M. bovis* is not a factor that can be changed by a farmer. However, this factor offers some insight into the possible duration of antibody detection post-infection and can be used as a proxy for self-selection bias. The results demonstrate that antibody testing of the BTM is a good way to determine whether *M. bovis* has recently been present within the lactating herd, and this cheap and quick tool should be utilised for regular testing on dairy farms.

The farmer's awareness prior to participating in the study was associated with the herd history of *M. bovis*, as discussed in Chapter 3. Although the confidence intervals were large due to one very small group, this association makes sense as farmers would be aware of a disease they have experienced in their herd. Both factors essentially act as a proxy to assess self-selection bias in the study.

4.5.3 Factors identified in the alternative multivariable models

Cow housing

An explanatory variable identified in one of the alternative models was the housing of the lactating herd. Herds that housed the lactating herd seasonally, semi-permanently, or opted for maximum grazing were less likely to be positive than herds that permanently housed some or all of the herd indoors. These results align with the findings of previous studies which reported that cows housed indoors for some or all of the year have higher instances of mastitis compared to outdoor housed cows (Sjostrom et al., 2019; Waage, Sviland and Ødegaard, 1998).

It is well documented that the type of housing presents different challenges of mastitis in dairy cows. Permanent grazing cows are at less risk of exposure to contagious mastitis pathogens as there is usually a lower stocking density compared to indoor housed cows. The lower stocking density means that there is less direct contact between cattle, thus a reduced risk of disease transmission. Conversely, in permanently housed cows, poor ventilation and higher humidity enables mastitic pathogens to thrive.

Seasonal housing where the cows are moved at certain points throughout the year will be exposed to the risks both indoors and outdoors. The advantage is that farmers can mitigate certain risks, such as keeping cows indoors during the wetter winter months while utilising the warm, dry weather for grazing. Though, the transition between indoor and outdoor housing can cause stress to the cows making them more susceptible to mastitis. Similarly, in semi-permanent housing, where cows may be housed during the evening and grazing during the day, udder health can be better than cows housed permanently (Washburn et al., 2002).

Ultimately, the association between housing and the presence of *M. bovis* reflects how intensive the system is, with housed herds tending to be larger and more intensively managed, providing optimal conditions for *M. bovis* spread.

All methods of housing have their advantages and disadvantages, but good management and hygiene are key to minimise the risk of *M. bovis*-mastitis across all systems. To date, there has been limited research into the risk of *M. bovis* and cow housing, though it is likely that the risks of *M. bovis* presence in different housing will be similar to other mastitis pathogens.

In the present study, farmers were not asked further detail on how the lactating herd were housed, such as the layout, space allowances and type of bedding. Nor were they asked to describe dry cow housing. This additional information could be explored in future studies to reveal further associations between cow housing and *M. bovis* prevalence.

Dairy bull and beef calf rearing

The majority of herds that reared their own dairy bull and beef calves tested antibody positive in their BTM. This is likely because there is an increased risk of disease transmission as there are more animals, and more groups of animals, in the farm. The potential for disease transmission will be influenced by the distance between animal groups. Where the lactating herd and youngstock are housed in close proximity and share common facilities, this will increase the risk of transmission compared to herds where the cows and youngstock are housed in separate sheds. Furthermore, as there is less of a market for dairy bull and beef calves, they are often fed waste milk and may receive poorer hygiene than replacement heifers, thus increasing the risk of disease spread, as discussed in Chapter 3.

The length of time that these calves are reared on farm will influence the risk of spread. For example, if they are reared until slaughter then there is more risk of disease maintenance and spread as there are more animals within the farm for longer compared to a farm that sells the calves before weaning. Although the duration of rearing these calves was captured in the questionnaire, due to small group sizes, the data had to be grouped to compare herds that rear dairy bull and beef calves to herds that don’t.

4.5.4 Factors not associated with the BTM prevalence of M. bovis

Herd size

There were a number of explanatory variables that were associated with the BTM results at the univariable level only, and some that were anticipated to be associated but were not at all. One factor not associated with the BTM antibody prevalence in the study was herd size. The biological explanations as to why herd size could be associated with the presence of *M. bovis* are an increased risk of transmission between individual cattle, and management and environmental effects related to herd size such as reduced observation of individual animals. Additionally, there is likely a higher turnover of cows in larger herds and thus an increased risk of introducing infected animals to the herd (Pinho et al., 2013). Previous studies reported that larger herds were at an increased risk of testing antibody positive. Herds greater than 120 were at 8.8 times more likely to have an antibody positive bulk milk sample (Hurri et al., 2022). Likewise, for every unit increase in size, Irish herds were 2.6 times more likely to test antibody positive (McAloon et al., 2022).

The absence of an association between prevalence and herd size in this study is in agreement with two studies that did not find an association with antibody-positive or PCR-positive BTM and herd size (Gille et al., 2018; Parker et al., 2017b). A similarity between the present study and that of Gille et al. (2018) is that the sample sizes were considerably smaller than that of Hurri et al. (2022) and McAloon et al. (2022), which were 3,144 and 1,313, respectively. It could be speculated that in the present study the sample size was not large enough to detect a difference in BTM positivity as a result of herd size. However, Fox et al. (2003) had a sample size of 164 herds yet detected an association between an increasing number of lactating cows and a higher probability of detected *Mycoplasma spp*. by PCR and culture.

The apparent prevalence of *M. bovis* in Scotland is much higher than estimates reported in other countries. It could be argued that although herd size was found to be a risk factor for *M. bovis* presence in dairy herds in other studies, as the disease is so prevalent in Scotland, all herds are at risk irrespective of their size. Though, it is difficult to compare the effects of different farming systems in different countries. There could be systemic differences in herd structures or

common management practices, some of which may be unique to the UK. Furthermore, biosecurity and biocontainment practices will likely differ between countries.

Additionally, due to the method of recruitment there is the potential that the association between herd size and the BTM antibody results was underestimated as farmers self-enrolled if they were interested in participating. This could have resulted in a biased study population which prevented the association being apparent.

Bought in cattle

Buying in cattle was not associated with positive bulk milk samples in the present study. Previous studies have identified the purchase of cattle as a risk factor for the presence of *M. bovis* (Fujimoto et al., 2020; Murai and Higuchi, 2019). Conversely, Haapala et al. (2021) reported that buying in cattle was significant in the univariable analysis but was not found to be significant in the multivariable model.

In the present study, the variable 'bought in cattle' was generated by combining the results of four separate questions asking farmers if they had bought in any of the following animals in the past 12 months: 0-12 month-olds, 12-24 month-olds, cows and bulls. This variable was used as a proxy for whether herds were open or closed.

There is no doubt that the purchase of cattle increases the potential for introducing any disease into a herd, and *M. bovis* is no exception. Introduction can occur via purchasing asymptomatic cattle which can be challenging to detect due to the intermittent shedding patterns of *M. bovis* (Biddle et al., 2003). A possible explanation for no association may be as *M. bovis* appears to be more prevalent in Scotland than other countries, all herds may equally be at risk, regardless of whether they purchased animals or not.

Other factors

One recent study observed an association between the presence of *M. bovis* antigen or antibodies in BTM and the use of a breeding bull (Gille et al., 2018). In the same study, herds that did not have a separate calving pen also had higher odds of being positive for *M. bovis* in their BTM. Both factors were included in

the questionnaire, but neither were associated with the BTM antibody results in the present study. The relationship between breeding practices and *M. bovis* is a generally understudied area, therefore it is not known how much of an impact different practices have on *M. bovis* presence and spread.

4.5.5 Lessons learned and limitations

There are many lessons that can be learned from conducting this longitudinal study on dairy farms in Scotland. Overall, the study was successful, and issues were minimised, though as with any study, there were some limitations.

Recruitment

Recruiting participants to a study can be challenging (Spratling, 2013): the recruitment approach influences the research findings (Newington and Metcalfe, 2014) and determines how much the results can be trusted (Jessiman, 2013). Therefore, carefully planning of a recruitment strategy is critical to the success of a research study.

Both the target and source populations for this thesis was dairy farmers in Scotland, who are notoriously busy people. Having knowledge of the study population helped formulate the inclusion and exclusion criteria used to select participants (Negrin et al., 2022). As there was no information available on the prevalence of *M. bovis* in Scotland at that time, it was decided to not narrow the inclusion criteria based on, for example, herd size and geographical location, to hopefully recruit a large number of farms to the study. Had it been decided to have narrower inclusion criteria, there may have been fewer eligible farmers volunteering to participate (Price et al., 2020). At least 88 farms were needed to estimate the prevalence of *M. bovis* in the BTM with 95% confidence and a precision of 10%. One hundred and eighty-one farms were recruited to the study, which was 93 more than required. This was a successful recruitment process as 21% of the total dairy farm population in Scotland participated in the study.

The first challenge was deciding how to recruit farmers to the study as this would influence the promotion. As *M. bovis* was a ‘hot topic’ at the time of planning the study, it was thought that by promoting the study directly to the dairy farmers, they could then contact us if they were interested. To minimise

selection bias as much as possible, the aim was to ensure that every dairy farmer in Scotland was aware of the study and had equal opportunity to participate. A press release was issued with SRUC, that was promoted in various farming and veterinary publications. An article was also published in the Scottish Farmer, a popular magazine that is read by most, if not all farmers in the country.

An alternative approach could have been to randomly, or systematically select dairy farms to participate and approach them, however there were confidentiality and data sharing issues. It was not possible to obtain the addresses of all dairy farms in Scotland, though thankfully Stuart Martin from the Scottish Dairy Hub was kind enough to help promote the study by posting study flyers with the mailshots that are sent to every dairy farm in Scotland. With the combination of the Scottish Farmer article, study flyers posted directly to farmers, and contacting vet practices and dairy companies asking them to promote the study to their clients, hopefully every dairy farmer in Scotland received some form of information about the study. Some selection bias may have occurred where vets could have encouraged clients with history of *M. bovis* infections to participate over herds with no *M. bovis* (or no suspected disease), leading to an overestimation of the BTM prevalence.

When allowing farmers to opt-in to the study themselves, this increased the risk of self-selection bias, as discussed in Chapter 3. Again, this could have led to an overestimation of the *M. bovis* BTM prevalence due to farmers who had experienced *M. bovis* in their herd being more likely to participate instead of those with no issues. As a proxy for determining if this was likely to have had an impact on the prevalence estimates, Question 14 of the BTM study questionnaire asked farmers if they were aware of *M. bovis* prior to participation. Not all participants were aware beforehand, and there was no association between their awareness and the BTM prevalence. It cannot be stated with certainty that there was no selection bias in the BTM study, however it was hopefully minimised with the methods of recruitment. Additionally, as presented in Chapter 3, Table 3-1, prior awareness of *M. bovis* was not associated with whether farmers enrolled in the study in the first or second recruitment periods.

Aside from the impact that the recruitment method had on the potential for bias, it also influenced the scope of the BTM study. To overcome the data sharing issues, one option was to approach milk recording companies and receive BTM samples anonymously from farms throughout Scotland. These samples would already be getting collected. The thesis study funds may have had to pay for the samples to be sent to us and provided the extra sampling tubes, though the overall costs may have been reduced as one box with all the BTM tubes would have been sent to a milk recording company four times throughout the year. Also, it would have been less labour intensive as individual sampling kits would not have been needed nor posted to each farm. Collecting samples via milk recording companies may introduce the potential for selection bias as around 72% of the total dairy population in Scotland are milk recorded (SDCA, 2022), and thus 28% would not be represented in the sample population.

Ultimately, it was decided not to have BTM samples collected via milk recording companies due to a variety of reasons. A key part of this PhD was to ensure that the findings were reported back at industry level to farmers and vets. Consequently, farmers' individual results were reported back to them and to their vet practice. It was also possible to provide a summary of all the results to each farmer so they could compare their results to the rest of the herds in the study. If samples were obtained from milk recording companies, there would have been no ability to contact farmers.

As the addresses of each participant were recorded, this also provided a spatial element to the results. The BTM results were not reported by region; however, this information was used to look at the representativeness of the study population. The study design would have also changed as it would not have been possible to conduct the questionnaire. Furthermore, repeated sampling would not have been possible anonymised BTM samples would have been provided. Finally, farms could not have been identified for the follow-on calf study.

Maintenance and dropout

Participant dropout, also known as attrition, is unavoidable in longitudinal studies (Jacobsen et al., 2021). As this was a longitudinal study spanning over one year, the aim was to ensure that dropout was minimised, and this was successful. The number of farms at each of the sampling points steadily dropped

down to 108, which was still 20 farms more than the minimum required, based on the sample size calculation (minimum of 88 farms needed).

For any kind of participant, farmer or not, it is important to ensure that their input and time required is minimal. Asking too much of study participants may deter them from continuing to participate. It was also made clear to the farmers that they could contact the author if they had any questions or concerns. On multiple occasions, farmers misplaced their sampling kit, and so a new kit was sent out to them. Several farmers did not send their second or third samples back, but they were contacted and rejoined the study for subsequent sampling periods.

It was also important to give something back to the farmers as a thank you for participating. They were already receiving free testing of their BTM, and their individual results were posted out to them after each sampling. It was also decided to send out *M. bovis* factsheets with the later three sampling kits. These provided information for farmers on *M. bovis*, how it spreads, and how to control it (shown in Appendix 3). The factsheets were created at a very low cost and were a simple gesture to farmers.

Building rapport with potential study participants improves their recruitment to studies (Negrin et al., 2022), and the same can also be said for maintaining their engagement in a study.

The use of technology in research studies

Technology now plays a bigger role in studies, with authors opting to use social media for study promotion and surveys being carried out via websites. It was important to the authors to ensure that all dairy farmers in Scotland would equally be able to participate in the BTM study. The use of survey websites was considered; however, this would have made participants complete the survey online and then receive a sampling kit in the post. This then may have led to missing data/results, or farmers dropping out as it was too time consuming. Similarly, online questionnaires can be difficult to navigate for some older generations, and with an older farming population in the UK it was better to make the study more accessible to all farmers throughout the country (Scottish Government, 2021). Paper copies made the questionnaire much more accessible

to all ages of farmers, and hopefully capture a more diverse range of farm management practices and structures. As discussed previously, this was also a consideration when planning the recruitment, enabling farmers to enrol online, by phone, or by post.

Impact of the COVID-19 pandemic

The COVID-19 pandemic had a significant impact on the BTM study, causing a delay of 5-6 months during the first UK lockdown. In February 2020, the study was promoted and around half of the total number of study participants had been recruited.

When the project was put on hold in March due to the pandemic, there was much uncertainty as to when it would be continued. All but two farms were retained from the first round of recruitment. These two farms were enrolled on the study but did not participate in the study as they had sold their business or cattle between March and August, and not because they were no longer interested in participating.

The halting of the protect may have also been the underlying reason for a small number of farmers that were interested in participating during the second round of recruitment choosing not to continue. It is unknown why four farms who were recruited in the second round of recruitment did not continue, though the three unknown BTM samples may have come from these farms.

Missing potential factors

There is always a chance that other, potentially important, factors are missed from data collection. The factors may become apparent after the study has been conducted. An example in this study is the presence of clinical disease within the study herds. In hindsight, it could have been a good additional factor to include in the questionnaire for a number of reasons. Firstly, it could be used as a proxy for self-selection bias. Similar to looking at the factors ‘herd history’ and ‘awareness’, if the majority of participants had observed clinical symptoms of *M. bovis* within their herd, then this could suggest that the study population was not representative of the total dairy herd population in Scotland, ultimately suggesting that there was in an overestimation of the associations observed between potential factors and the BTM results.

Secondly, this variable could have been compared to the BTM antibody results to determine if there was an association between higher OD levels and the appearance of clinical symptoms. However, the presence of clinical *M. bovis* disease was not included in the BTM as it was beyond the scope of the study. Furthermore, as discussed previously, the clinical signs are not specific to *M. bovis* alone, and there is the existence of asymptomatic animals.

A comprehensive questionnaire could have been sent to farmers addressing further details on topics such as the rearing of youngstock, milking, housing and health management. Though, the questionnaire was kept brief to not discourage farmers from participating with an overwhelmingly long questionnaire, and to make a start on identifying potential risk factors in Scottish dairy herds which will hopefully be expanded on in future research.

Using the first BTM sample as the outcome

The risk factor analysis was conducted using only the ELISA result from the first BTM sample. This approach was chosen because the questionnaire was completed by farmers at the same time as they collected their initial BTM sample. Changes within the herd that occurred afterward were not documented, thus any associations identified could be under- or over-estimated. While farmers were asked to report relevant changes when collecting subsequent samples, most did not provide additional information. Among the few who did, most focused on changes in vaccination status. Unless farmers explicitly stated ‘No changes’ or similar, it cannot be assumed that there were no changes in the herd between sampling points.

Additionally, at each sampling point, the number of participating herds decreased, with the first sampling point having the largest cohort. Detecting associations requires a sufficiently large sample size, particularly for smaller associations (Shreffler and Huecker, 2024), making it more appropriate to base the analysis on the first BTM sample results.

Sample size

To estimate the prevalence of *M. bovis*, the sample size calculation determined that a minimum of 88 farms were needed in the study. There were 181 farms included in the risk factor analysis, which was above the minimum requirement of 88 to estimate the prevalence. There is however a possibility that the sample

size was not large enough to detect associations between the explanatory variables and the first BTM antibody results. Although no associations were found between most of the explanatory variables and the outcome variables in the present study, it does not mean that the associations do not exist. When a factor has a strong effect on an outcome, only small sample sizes are required to identify these associations, whereas when the factor has a small effect on the outcome, a much larger sample size is required to observe the association. There is a possibility that some true associations were missed in the present study due to the sample size, for example the herd size as mentioned earlier.

Representativeness

The mean herd size of the study population (number of cows) was 245 which was slightly greater than the mean herd size of the total dairy population in Scotland which was 209 (SDHA, 2021). It was difficult to obtain data on all dairy herd in Scotland to compare the distribution of herd sizes in Scotland and the study population due to data protection. Consequently, only point estimates of the mean herd size of the study population could only be compared to the average size of all dairy herds in Scotland. If the study population was greater or smaller than the average herd size, there would be a risk of under-or over-estimating the prevalence of *M. bovis* and the factors associated with the prevalence.

The representativeness of the study population was also assessed by comparing the geographical distribution of study herds to all dairy herds in Scotland. The proportion of study herds in each region was very similar to that of the total dairy herd population in Scotland. The farm postcode was used to assign the farm to a county, however, as discussed in Chapter 3, it was difficult assigning farms to the correct region as some postcodes were spread across two separate regions. Furthermore, it is not known exactly how the location of all Scottish dairy herds was categorised into each region in the reference data. Therefore, it is possible that the proportions of study herds, and of the total dairy herd population, were slightly different to the true picture.

In summary, the key to conducting a successful longitudinal study involving farmers is good communication and ensuring that there is the time available to maintain their participation. Farm population data can be difficult to obtain due to data sharing issues which creates a challenge in assessing the

representativeness of the study population. This issue cannot be avoided, however it can and should be acknowledged.

4.6 Conclusions

The results of this study suggest that the majority of herds in Scotland may be exposed to *M. bovis*, highlighting the importance of adopting good biosecurity practices to prevent the introduction of *M. bovis* into a herd. Negative herds are extremely valuable and should be protected.

There was evidence of recent exposure to *M. bovis* in around three quarters of herds at each of the four sampling points. Furthermore, with over 80% of herds testing positive at least once throughout the study, this highlights the importance of adopting good biosecurity practices to prevent the introduction of *M. bovis* into a herd. The BTM prevalence of *M. bovis* by PCR testing was extremely low in the study, with only four herds testing positive. This suggests that the use of PCR tests to detect the presence of active infection in the BTM needs to be supported by antibody testing, and within-herd testing of individual cows.

A high prevalence does not necessarily translate to high instances of clinical disease, therefore this is an area that requires more research. Further studies are required to determine the true impact of *M. bovis* within-farm in Scotland.

At the time of writing this thesis, *M. bovis* was not considered a pathogen of significance in Scotland, hence why there were no national control or monitoring strategies. However, the UK Cattle Expert Group published a report in 2018 which summarised the knowledge gaps that needed to be addressed on *M. bovis* in the UK (UK Cattle Expert Group, 2018). One key area discussed was an estimation of the prevalence of *M. bovis*, which would support the development of disease eradication programmes. The findings of this study may initiate conversations surrounding the need for *M. bovis* monitoring or eradication programmes in Scotland, and the rest of the UK.

Although the present study did not have enough predictive power, it has been highlighted that buying in cattle is a potential risk factor for the presence of *M. bovis* antibodies in BTM. Introducing cattle from outside of the herd increases the potential for the introduction of any pathogen into the herd. This is why the

adoption of good biosecurity should be continued within the industry. Likewise, herds that have had a recent *M. bovis* diagnosis will likely test antibody positive in the bulk milk as antibodies are thought to be shed for long periods of time following active infection. The interesting takeaway from this study is that if a farmer suspects that *M. bovis* has been present recently, then they are probably right.

Ultimately, it is apparent that *M. bovis* may be more prevalent in Scotland than previously thought, and the next steps should be to determine how much of an impact this disease is having within farms.

Chapter 5 Classification of *Mycoplasma bovis* infection status of Scottish dairy farms – a longitudinal study

5.1 Introduction

5.1.1 Background on M. bovis

M. bovis is widely recognised as a major pathogen causing a range of clinical symptoms including bovine respiratory disease, arthritis and mastitis in cattle worldwide. Infections with *M. bovis* cause significant economic losses resulting from poor growth rates, reductions in milk production/yield (Timonen et al., 2017), and increased mortality and morbidity (Nicholas, 2011). In addition to causing mastitis and respiratory disease, secondary symptoms of *M. bovis* infections include otitis media, arthritis, keratoconjunctivitis, and reproductive disease. Upon exposure, some cattle remain asymptomatic though are still carriers of the pathogen and are a potential source of infection to the rest of the herd. *M. bovis* can be transmitted readily within a farm via milk and colostrum, semen, as an aerosol, and on fomites such as feeding equipment (Maunsell et al., 2011).

Although there has been increased awareness of *M. bovis* in the British veterinary and scientific communities, there are still huge gaps in the overall understanding of this pathogen, (Calcutt et al., 2018). One of the major challenges with *M. bovis* is that the organism itself lacks a cell wall and does not synthesise folic acid, which are two important areas that are targeted in antimicrobial therapies, thus limiting treatment options (Maunsell et al., 2011). Though *M. bovis* is susceptible to antimicrobials that target protein or DNA synthesis, some strains of *M. bovis* are developing resistance to the few antimicrobials that can be used against it (Lysnyansky and Ayling, 2016). Improving our understanding of the presence and persistence of *M. bovis* infections within dairy herds could support the development of better herd-level disease monitoring and reduce reliance on antimicrobial treatments. Furthermore, with the global rise of antimicrobial resistance of *M. bovis*, it is

imperative to develop robust national and herd-level control and surveillance strategies.

Historically, diagnosis of *M. bovis* heavily relied on culture methods which proved slow and had reduced sensitivity, particularly when polymicrobial infection was present (Nicholas and Baker, 1998). The high sensitivity, reduced processing time and cost of PCR has improved the diagnosis of *M. bovis*, however due to the intermittent shedding nature of this pathogen, the sensitivity of these tests may be reduced. BTM testing is frequently used as a complementary diagnostic tool to detect if there is evidence of recent exposure in herds by testing for *M. bovis* antibodies.

The impact of *M. bovis* in Scotland is not known, though anecdotally, *M. bovis* is thought to be present on a high proportion of dairy farms throughout the country, with farmers and veterinarians reporting outbreaks of BRD in youngstock. The number of *M. bovis* diagnoses made on submissions to the Great Britain (GB) Veterinary Diagnostic Network has been steadily increasing over the past decade (GB Veterinary Diagnostic Network, 2023). As discussed in Chapter 1, the reason for this increase may be due to a true increase in the prevalence of *M. bovis*, increased awareness of the pathogen, or changes to diagnostic testing.

By estimating the herd-level prevalence of *M. bovis*, this would be the first step towards building the overall picture of this disease in Scotland and could support the future generation of national and farm-level control strategies.

5.1.2 Disease surveillance and health schemes in Scotland

Surveillance can be defined as a system that collects data on the frequency and distribution of disease in a population for analysis and interpretation, which initiates interventive or preventive action (Torrence, 1997).

In terms of disease occurrence, surveillance contributes towards the early detection of outbreaks within a population. Many types of surveillance exist, though two commonly used approaches are active and passive. Active surveillance is the targeted collection of data from a population for a specific health condition or disease (Thrusfield et al., 2018). This type of surveillance can often be formulated as part of a scientific study carried out by a research

group where there is particular interest in the health condition and therefore is typically labour-intensive and costly.

On the other hand, passive surveillance is the collection of data within an organised system (MacDonald et al., 2021), such as routine post-mortems carried out at veterinary disease surveillance centres throughout the country, which means this data is often biased (Thrusfield et al., 2018). This method of surveillance still requires a considerable amount of input (Torrence, 1997).

Herd screening is a monitoring activity where a subset of, or the total herd is sampled to determine the disease status of the herd. A random or systematic selection process will be used to sample from the population to detect asymptomatic or subclinical animals (Thrusfield et al., 2018). One or multiple groups of animals may be targeted of different ages, sex, or stage in production.

With the UK leaving the European Union, marketability of produce is of increasing significance for British farmers to be competitive in the global market. Furthermore, there is increasing public demand for traceability and transparency of food production (Zhang et al., 2020). Evidence of high welfare standards and freedom from disease through accredited health schemes provides consumers with confidence that their food has been produced to a high standard.

In Scotland, there are a handful of voluntary health schemes farmers can join which require them to undertake regular screening for a range of diseases. The purpose of health schemes at a national level are to monitor, control and/or eliminate infectious endemic diseases of cattle throughout the country. At herd-level, health schemes are a means for farmers to improve the overall health of their herd and identify ways to control and eradicate disease.

Participation in herd health schemes increases productivity and animal welfare, and reduces the financial burden from treatment, losses, and diagnostic testing. Furthermore, participating in a health scheme can provide herd accreditation, sometimes providing proof of freedom from disease, enabling farmers to purchase cattle from other accredited herds and reduce the risk of disease reintroduction.

In the UK, including Scotland, there are various health schemes that are overseen by the certifying body CHeCS (Cattle Health Certification Standards). Enrolment in these schemes is not mandatory, however it is highly recommended

to improve the overall herd efficiency. Herds are screened for diseases that are the most significant single-agent infectious diseases in beef and dairy herds in the country such as BVD virus, infectious bovine rhinotracheitis (IBR), Johne's disease, leptospirosis, Neospora, and bovine tuberculosis (bTB). The economic consequences of these diseases being present within a herd can be devastating due to veterinary costs, culling, and reduced production (Andrés-Lasheras et al., 2021; Orpin and Esslemont, 2010). A proportion of cattle exposed to these diseases may remain asymptomatic, having the ability to consistently infect the rest of the herd and remain undetected unless tested. Frequent screening of individuals within a herd increases the likelihood of detecting these asymptomatic individuals which can then be removed from the herd (Thrusfield et al., 2018). The diseases in the voluntary health schemes have the ability to transmit readily within and between-farm, and some are zoonotic.

Other forms of screening may also be formulated independently with the farmer's vet to monitor for specific diseases, or clinical symptoms such as mastitis, that have recently been or are currently challenging the herd. Cattle may also be monitored throughout the grazing season to assist in decision making for anthelmintic use.

5.1.3 Mycoplasma bovis surveillance and monitoring

In Scotland, and the UK as a whole, there are currently no health programmes that incorporate the screening of herds for *M. bovis*, nor is there a national control strategy. The prevalence of *M. bovis* and its impact on the Scottish dairy (and beef) industries is not known (UK Cattle Expert Group, 2018). Information on the prevalence of *M. bovis* in Scotland could be used to support the potential development of a national *M. bovis* monitoring programme.

Arguably, many of the agricultural systems seen throughout Scotland differ greatly to the rest of the UK, and this is evident in the Scotland-specific approaches to national disease control via health schemes such as bTB and BVD. Therefore, establishing a Scotland-specific approach to monitoring and controlling *M. bovis* may be appropriate, though this would still have to align with practices in the rest of the UK.

In New Zealand (NZ), after what was believed to be the introduction of *M. bovis* into the country in 2017, a national eradication scheme was established in 2018

which involved mass testing and culling of infected herds (Laven, 2019). The eradication scheme required close coordination between government agencies, dairy farmers and vets, and involved testing BTM and individual animals. Once *M. bovis* was confirmed within the herd, they were depopulated. This scheme had a significant economic impact on the NZ dairy industry and negatively affected the mental health of many farmers (Noller et al., 2022).

The main clinical presentations of *M. bovis* in NZ is different to that of the UK. In NZ, mycoplasma-mastitis is the primary issue, causing significant losses in milk production from clinical disease. Conversely in the UK, calf pneumonia is the most commonly reported issue associated with *M. bovis*. The differences between the situation in NZ and the UK may be due to different strains of the pathogen and the structure of the dairy industries.

If *M. bovis* appears to be highly prevalent in Scotland and had a significant impact on welfare and production, this could encourage more herd screening for *M. bovis*. To develop a potential surveillance scheme or national control strategy for *M. bovis*, a method for classifying herds based on their *M. bovis* disease status would first be required.

5.1.4 Classification of herds

Classification, or categorisation, is the act of sorting ‘units’ into groups with others that possess similarities (Gordon, 1987), wherein a unit could be an individual animal, a group of cattle, or a herd.

Categorisation is used on dairy farms for housing cattle by age to ensure that the type and volume of feed provided is appropriate. Likewise, cattle may be housed by sex, breed, or stage in lactation. Cattle may be categorised into ‘sick’ or ‘healthy’ by the farmer and/or vet depending on the appearance of clinical signs or diagnostic test results for a specific disease or health condition (Thrusfield et al., 2018). This categorisation enables resources to be focused on the sick animals which may involve segregation, treatment, or culling, and preventative action can then be taken on the healthy animals including vaccination.

At herd-level, herds can be categorised based on the farm type and production, such as organic vs non-organic, intensive vs extensive, or indoor vs outdoor. Herds are also categorised based on disease status in herd health schemes as

described Section 5.1.2. Knowledge of herd-level disease status can help form national surveillance and control strategies by identifying regions or areas with higher disease prevalence or incidence. Furthermore, herd-level risk or preventive factors can be identified that are associated with the presence or absence of disease, respectively.

Depending on the purpose of categorisation, the definition of the categories may be strict where herds belong to only one group, or the classification may be more lenient where herds can belong to multiple categories (Cormack, 1971). A herd is defined as organic if fertilisers and pesticides are not used. In this example, a herd cannot be classified as 'partly' organic, and so the herd is either organic or non-organic. On the other hand, the calving period may be less easy to define, for instance, a farmer may state that the calving period of the herd is in the summer, though it happens to tail off into the autumn. Therefore, by definition, this is not strictly a summer calving herd, as it could be classed as both a summer and autumn calving herd. Additionally, what one individual classes as summer may not be the same as another person, unless the seasons are clearly defined.

Categorisation of herds based on *M. bovis* disease status must be biologically plausible and be of use to the industry (Everitt et al., 2011). In other words, what benefit would it be to farmers and vets to categorise dairy herds in Scotland based on their *M. bovis* disease status, and how can this information be utilised? At the individual herd level, farmers would benefit from knowing whether their herd has the disease present and if the disease prevalence is changing, i.e. increasing, decreasing, or remaining stable. Classifying the *M. bovis* disease status of dairy herds could be applied to determine why positive herds are at more risk of disease than negative herds. Furthermore, categorising herds based on whether they are positive or negative and reporting the number of herds in each category would support policy makers to decide if a national control strategy for *M. bovis* is required. Moreover, this knowledge could be used to predict future disease trends.

The question(s) behind the categorisation of herds by *M. bovis* disease status must be clearly defined as this influences the method of categorisation. If the aim is to distinguish between herds that are positive or negative, then herds

would be categorised based on the diagnostic test cut-off. Alternatively, there may be a need to categorise herds by disease trend, which could be based on disease changes over time.

Different methods could be applied to classify the *M. bovis* disease status of a herd based on BTM results; these could be simpler, manual methods of categorising herds or more statistically complex model-based clustering.

5.1.5 Computational methods of clustering

Clustering definitions

A cluster in a dataset is a series of events or cases that are closely related in time and/or place. Model-based clustering is a means of identifying any patterns and subgroups of observations within a dataset (Everitt et al., 2011), particularly similarities and differences that may not otherwise be obvious in a heterogenous population. Each case within the dataset is grouped into clusters which contain cases that are similar to each other and dissimilar to those in other clusters. Different types of cluster analysis exist such as hard clustering wherein a single data point is placed into only one of the existing clusters. Hard clustering methods do not account for the fact that individuals may share traits with multiple subgroups in the population and therefore may potentially belong to multiple clusters (Bolin et al., 2014). Alternatively, soft clustering is when a single datapoint can be a member of more than one cluster at a time. The choice of using hard or soft clustering methods will depend on the question to be addressed. If the purpose is to identify distinct categories within a population, for example, classifying herds based on whether they are positive or negative, then hard clustering would be the most appropriate approach as a herd cannot be partly positive or negative. An example where soft clustering may be applicable is for categorising farms by production type, i.e. a farm that has both beef cattle and sheep.

K-means clustering

K-means clustering is one of the fastest, simplest and most popular methods of non-hierarchical clustering used to group datasets (Jahwar and Abdulazeez, 2021). Individuals are categorised into a predefined number of clusters of equal

variance, minimising the inertia which measures the coherence of the clusters (Cui, 2020). This method of clustering does not require there to be a training or reference dataset to compare the clusters which is why this is a popular method of clustering (Jahwar and Abdulazeez, 2021). Additionally, k-means clustering has a tendency to create equal clusters, i.e. clusters will have similar numbers of data points and radius (MacQueen, 1967).

K-means clustering involves three stages. First, once the value for k has been prespecified, the initial centroids, or means, are selected based on all individuals in the dataset (MacQueen, 1967). The subsequent two stages are repeated in loops. Individuals are assigned to the nearest centroid, and then new centroids are generated by calculating the mean value of all individuals assigned to each previous centroid. The difference between the old and new centroids are calculated, and this process is repeated until the centroids do not move significantly (Genolini and Falissard, 2010).

In addition to clustering subgroups of individuals based on a single point, k-means clustering is used in the analysis of longitudinal data to identify subgroups based on trends over time (Usami, 2014). This information can be used to identify typical patterns that could be expected, for example, the antibody shedding patterns for infected individual cows. Similarly, clustering can be used to predict future trends.

Applications of clustering

Cluster analysis has applications in many different fields including medicine to group patients based on their medical prognosis, marketing to cater advertising to the most appropriate audiences, social sciences, and city planning to categorise houses based on their value (Adolfsson et al., 2019).

The use of clustering methods is relatively novel in the field of veterinary sciences, with only a handful of papers published to date. Clustering methods were first applied to compare methods of categorising herds based on longitudinal changes in BVD antibody test values (Eze et al., 2019). This study clustered herds based on the shape, trend, and magnitude of their BVD trajectories. Oehm et al. (2022) used k-mode clustering to cluster German dairy farms based on their BTM *Fasciola hepatica* and *Ostertagia ostertagi* antibody

status, and some milk parameters. More recently, researchers used hierarchal cluster analysis to identify two clusters of bovine respiratory disease outbreaks in Spain (Calderón Bernal et al., 2023).

5.1.6 Study aims

The study had two aims: to observe how the BTM prevalence of *M. bovis* changed over time, and to explore methods of classifying herds based on their BTM *M. bovis* disease results.

5.2 Methods

5.2.1 Longitudinal bulk tank milk prevalence study

Study design

A one-year longitudinal BTM prevalence study was conducted on Scottish dairy farms with four sampling points roughly three months apart. Participants were asked to submit BTM samples at each of the four sampling points and complete a sample submission form. A short questionnaire on general herd management practices was completed with the first samples.

Recruitment

Dairy farms were recruited as described in Chapter 2. Briefly, the study was promoted via veterinary and farming publications to increase awareness to dairy farmers throughout Scotland. Promotional flyers with information on the study were posted to every dairy farm in the country. Farmers had the option to express their interest in the study by various routes, phone, email and post. Once interest was received, the first sampling kit was posted to the farm containing sampling instructions, milk tubes, and relevant documents including a consent form.

Maintaining participation

To retain participants in the study, individual farm results were posted to each participant after every sampling point, accompanied by a *M. bovis* ‘factsheet’

for participants to read at their own leisure. The primary researcher aimed to keep in regular contact with participants throughout the study reminding them when to expect and return sampling kits. Participants were also sent a summary of their own results and the overall preliminary results.

Sample testing

BTM samples were tested using the Applied Biosystems VetMAX *M. bovis* PCR kit for the presence of active infection and the ID Screen® *Mycoplasma bovis* indirect ELISA kit to detect the presence of antibodies. A full description of the methods is shown in Chapter 2.

Prevalence definitions

The prevalence of active *M. bovis* infections (PCR test) and of antibodies to *M. bovis* (ELISA test) were estimated at each of the four sampling points. The prevalence of active *M. bovis* infection was calculated using the following equation.

$$Prevalence = \frac{Total\ number\ of\ PCR\ positive\ samples\ at\ sampling\ point}{Total\ number\ of\ samples\ collected\ at\ sampling\ point}\ X\ 100$$

The following equation was used to calculate the prevalence of *M. bovis* antibodies.

$$Prevalence = \frac{Total\ number\ of\ antibody\ positive\ samples\ at\ sampling\ point}{Total\ number\ of\ samples\ collected\ at\ sampling\ point}\ X\ 100$$

To correct for an imperfect ELISA test, the true prevalence was calculated using the test sensitivity and specificity of 95.7% and 100%, respectively, and using the Wilson confidence interval of 95% (Sergeant, 2018a).

The total number of PCR and ELISA positive samples across all four sampling points were also calculated.

5.2.2 Categorisation of farms based on infection status

To classify herds based on their *M. bovis* infection status, various approaches were trialled based on the PCR test results, the ELISA test cut-off, and the ELISA test optical density values.

Based on BTM PCR

Only four farms tested positive by PCR for the presence of *M. bovis* DNA in BTM. Farms were categorised as being positive for active infection at each of the four sampling periods.

Based on BTM prevalence

Farms that sent in three or four BTM samples as part of the *M. bovis* BTM prevalence study were included in the farm status categorisation analyses. In total there were 133 farms included in this analysis. The mean OD% was calculated for farms that sent in three samples (n=27) and included in the data as a fourth OD value for the missing result.

Using the optical density cut-off:

Farms were categorised initially by looking at the results as a binary result, i.e. positive or negative. The cut-off for positivity was defined as ≥30 as recommended by the test kit manufacturer.

Five trends were observed: consistently positive, consistently negative, seroconversion, sero-reversion, and a final category 'other' containing farms that changed back and forth between positive and negative. After looking at the different categories, for the purpose of analysis, three distinct categories were defined: consistently positive, consistently negative, and transitional (Table 5-1). The transitional category contained all farms that seroconverted, sero-reverted, the 'other' group.

Table 5-1: Categories created for herds based on the BTM ELISA test cut-off

Category	Definition 1	Definition 2
Consistently positive	Positive in all samples	Positive

Category	Definition 1	Definition 2
Consistently negative	Negative in all samples	Negative
Seroconversion	Start negative and end positive	Transitional
Sero-reversion	Start positive and end negative	
Other	Results back and forth between positive and negative	

Using raw OD data:

Farms were categorised based on the unit of change between the OD value of sample one and sample four. The difference between sample one and four was calculated for each farm by taking the result of sample 4 and subtracting the result of sample 1. Farms were categorised into either: change of 10 or less, increased by more than 10 units, or decreased by more than 10 units.

Model-based clustering:

The optical density trendlines for all 133 farms were graphed and it was apparent that there was huge variation in the OD% trends among the study farms, as shown in Appendix 6, Figure A6-2. A plot illustrating this variation is shown below, Figure 5-1.

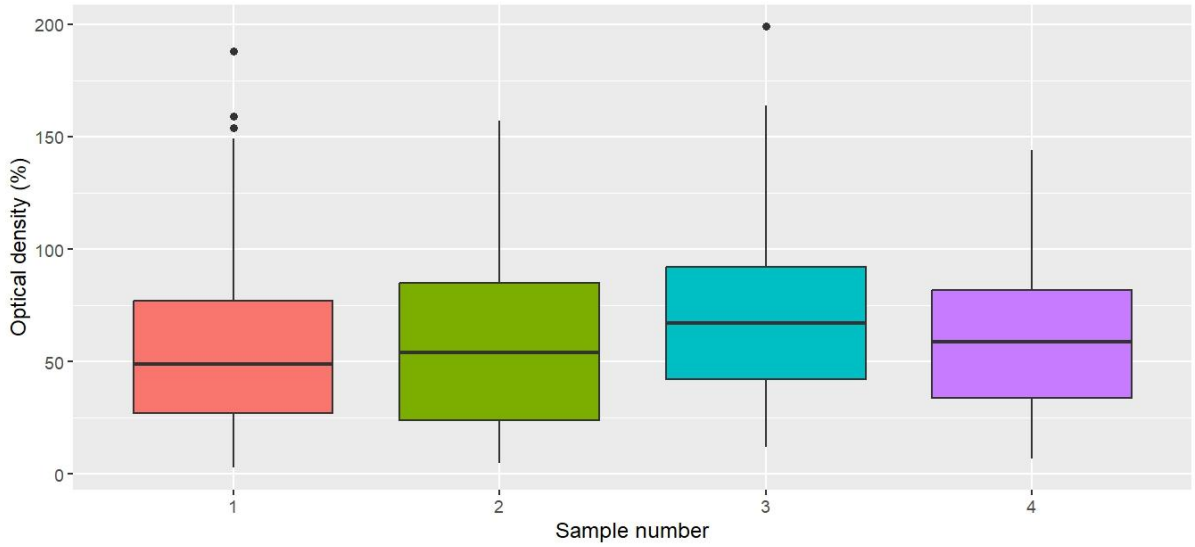


Figure 5-1: Boxplot showing the variation in OD% for each of the four sampling points.

On further observation of the data, there appeared to be three general trends: farms with a decreasing slope, farms with relatively stable slopes, and farms with increasing slopes.

A simple linear model was fitted to the mean antibody OD trajectory for each farm as described by (Eze et al., 2019), using the following equation:

$$y_{it} = \alpha_i + \beta_i t + \varepsilon_{it}$$

Where y_{it} is the mean antibody OD value in the farm i at time t. The intercept and slope for farm i are represented by α_i and β_i, respectively. The random error is ε_{it}.

The evident difference in steepness of slopes, or the rate of change, is shown in the histogram (Figure 5-2).

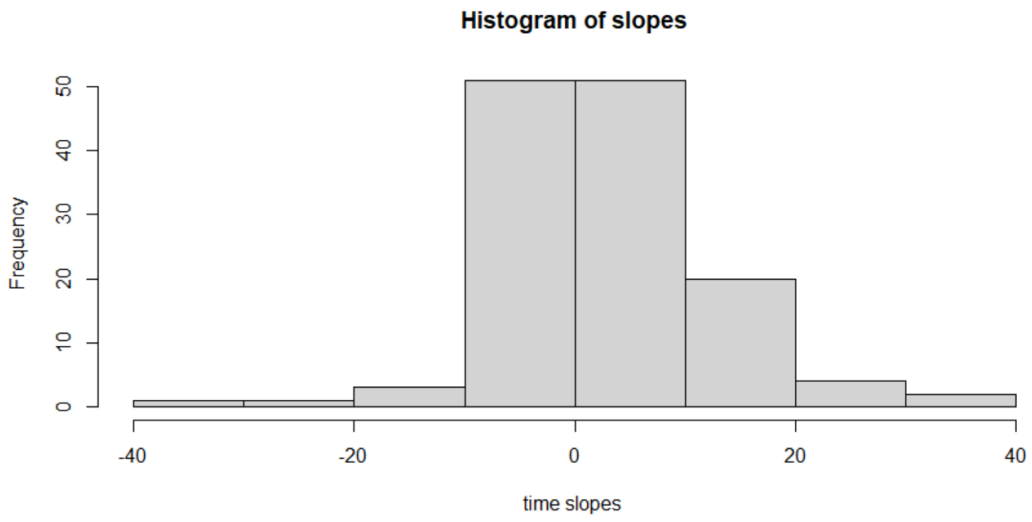


Figure 5-2: Histogram of the time slopes generated from the optical density trendlines

This proved that there was variation within the dataset, therefore clustering could be used to group farms based on their BTM OD trends.

Longitudinal k-means analysis (LKMA) was the chosen method for clustering the farms based on their four BTM optical densities. Various clustering packages in R were used to explore different methods of LKMA clustering of the farms: *Mcclust*, *Kmeans* and *akclustr*. The function a*kclustr* from the package *akmedoids* was the final choice. The number of clusters must be predefined when computing k-means clustering, therefore it was decided to trial between 3 and 8 clusters. The Calinski-Harabasz (C-H) index is a criterion in *akclustr* that can be used to compute the optimal number of clusters for the dataset. The C-H index compares the inter- and intra-cluster distances to determine the optimal

number of clusters. This criterion is appropriate when the ground truth labels are not known, i.e. there was no reference data to compare the clusters to. Once the optimal number of clusters were defined, the function was re-run with the chosen value of k and the farm assignment to clusters was extracted.

The cluster assignment of farms was compared to the categorisation of farms based on the unit of change from sample one to sample four, to observe the level of agreement between the two methods.

Chi-squared testing was then used to look for associations between the cluster assignment of farms and the farmers' responses in the questionnaire. Fisher's Exact tests were used where the group sizes were less than 5.

5.3 Results

5.3.1 Summary of BTM results

The longitudinal BTM results from both the PCR and ELISA testing are presented in Chapter 4. To summarise, four herds tested positive by PCR, three tested positive in one BTM sample and one herd tested positive in two consecutive samples. The majority of herds tested antibody positive in all four sampling points: 76%, 71%, 83% and 79%.

As mentioned in Chapter 4, there were seven herds that reported using a vaccine against *M. bovis*. The longitudinal BTM results of these herds are presented in Appendix 6. In all seven herds, the OD% was consistently above the test cut-off of 30%. Six of the herds completed three or four sampling points and were therefore included in the categorisation work discussed below. One vaccinated herd was not included as they only completed two sampling points.

5.3.2 Categorisation of farms based on bulk tank milk PCR results

As there were only four herds that tested positive by PCR for the presence of *M. bovis* DNA in BTM, herds were not categorised based on this test result.

5.3.3 Categorisation of farms based on the prevalence of M. bovis antibodies in the BTM

Using OD cut-off

The mean OD% trends for the five initial categories are shown in Figure 5-3. Each category was observed individually.

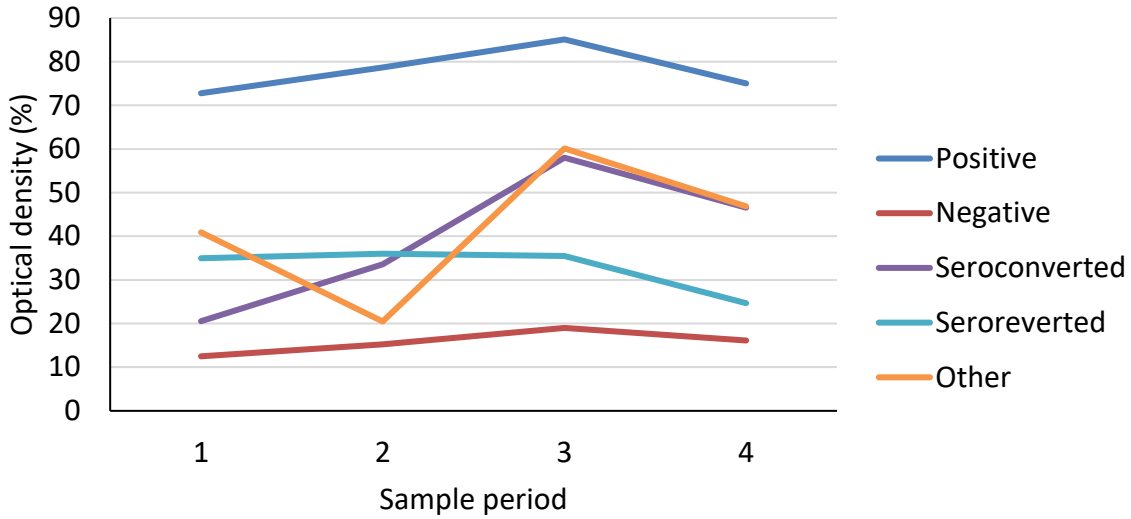


Figure 5-3: Mean ELISA OD% trends for the five classifications based on the test cut-off

The optical density trends for all consistently positive herds are shown in Figure 5-4. In total 81 herds tested consistently positive for the entire duration of the study.

Upon observation of the data, it was evident that there was a great deal of variation in OD% among the consistently positive herds, with some remaining around the lowest positive value of 30% and the highest OD value at 199%, with a range of 169.

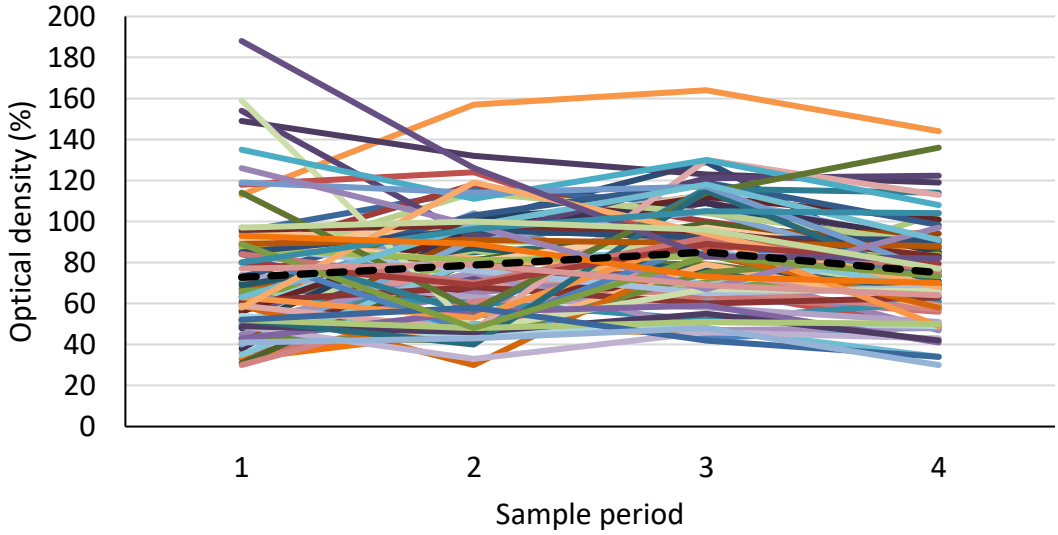
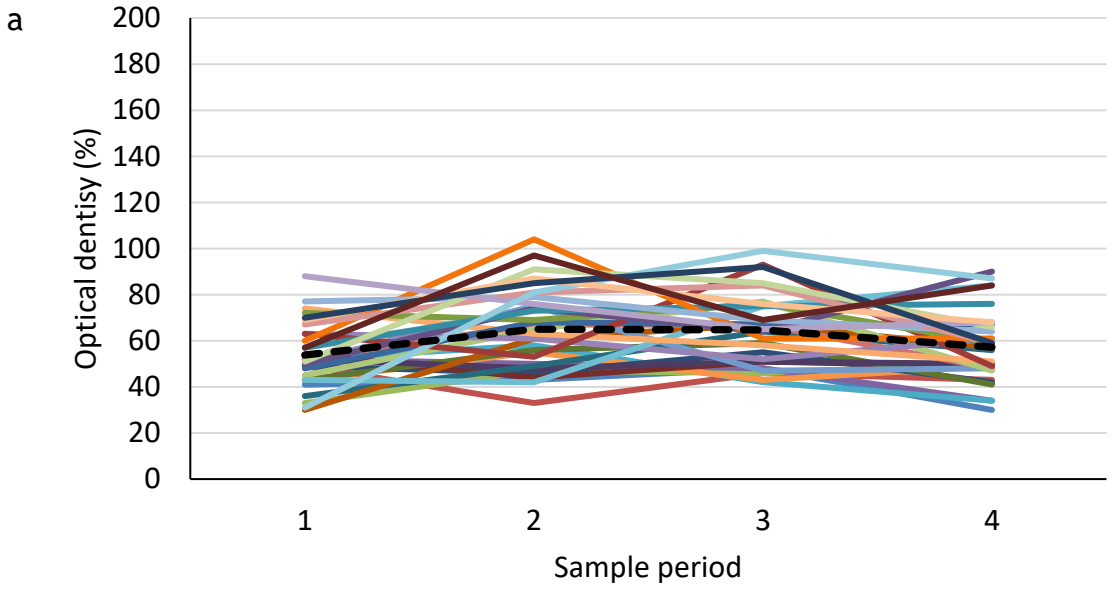


Figure 5-4: ELISA OD% results for all eighty-one herds in the consistently positive category. Mean OD at each sampling point is represented by the black dotted line.

As there was variation within the consistently positive herds, this group was further categorised into low (n=41) and high (n=40) positive herds (Figure 5-5).



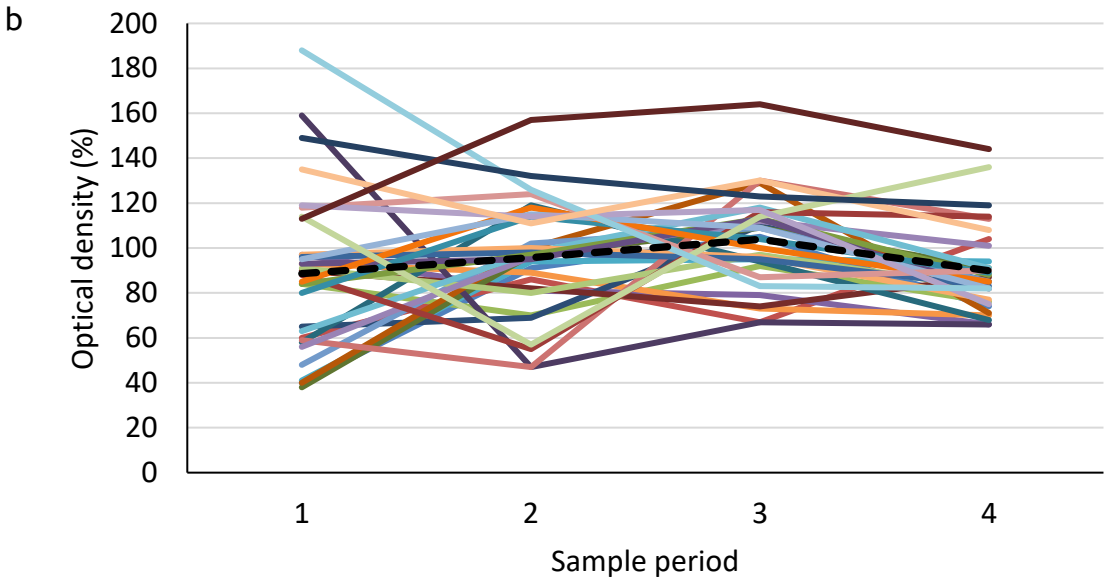


Figure 5-5: ELISA OD% results for all eighty-one herds in the consistently positive category, split into (a) low positive and (b) high positive. Mean OD at each sampling point is represented by the black dotted line.

The mean OD% was calculated for each farm and ordered from lowest to highest and divided into two groups based on the mean OD%, a low and a high positive group. The mean OD% trends for each of the low and high positive categories are shown in Figure 5-6.

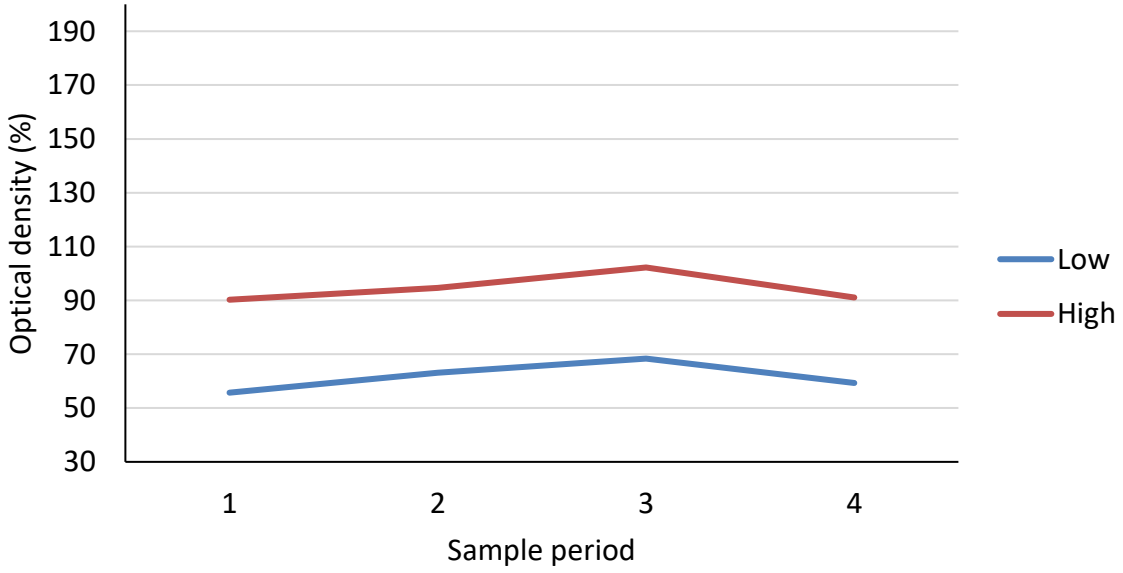


Figure 5-6: Mean ELISA OD% trends for the low and high positive herds based on the test cut-off

Twenty farms tested consistently negative for all four BTM samples (Figure 5-7). The minimum OD value in this category was 3% and the maximum was 27%. The results suggest that these herds had not experienced recent exposure to *M. bovis*

as there were no detectable levels of *M. bovis*-antibodies being shed in the bulk tank at the time of sampling.

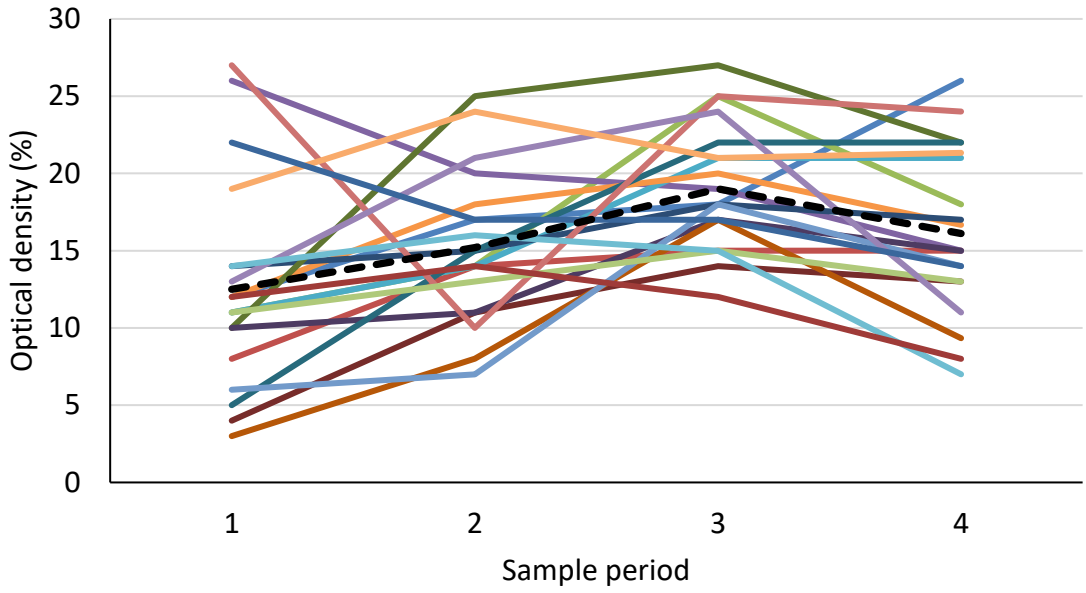


Figure 5-7: ELISA OD% results for all twenty herds in the consistently negative category. Mean OD at each sampling point is represented by the black dotted line. Full y-axis range of 200% OD not used to enable clear observation of OD trends in the negative group.

Twelve farms seroconverted from negative to positive, suggesting that *M. bovis* may have been introduced during the study period (Figure 5-8). The minimum OD value in this category was 8% and the maximum was 130%.

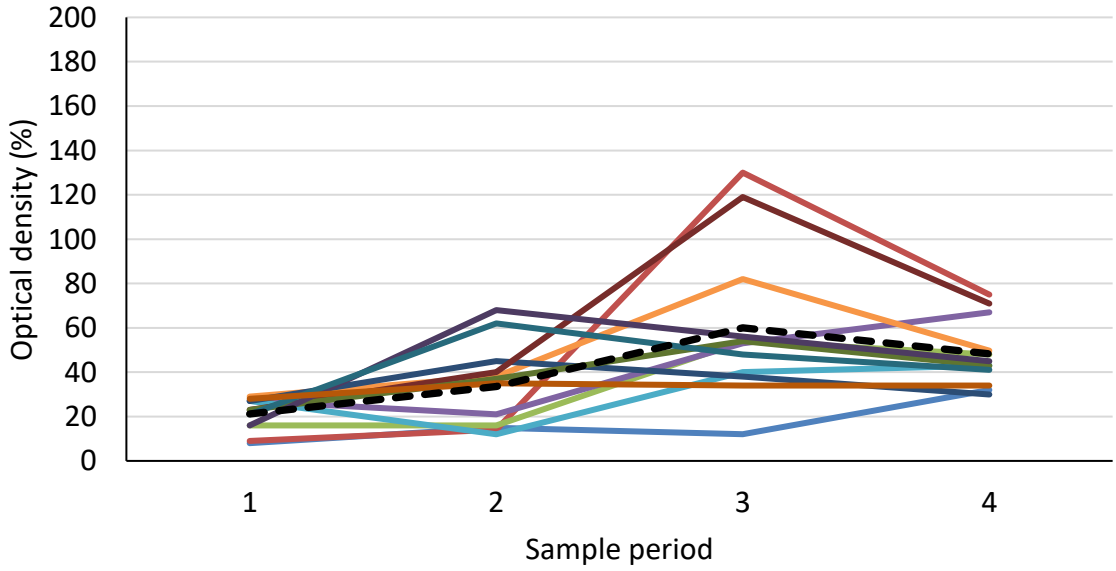


Figure 5-8: ELISA OD% results for all twelve herds in that seroconverted. Mean OD at each sampling point is represented by the black dotted line.

Only two farms sero-reverted from positive to negative throughout the study, this would imply that these herds may have recovered from recent *M. bovis* infection (Figure 5-9). The maximum OD value was 54% and the minimum was 18%.

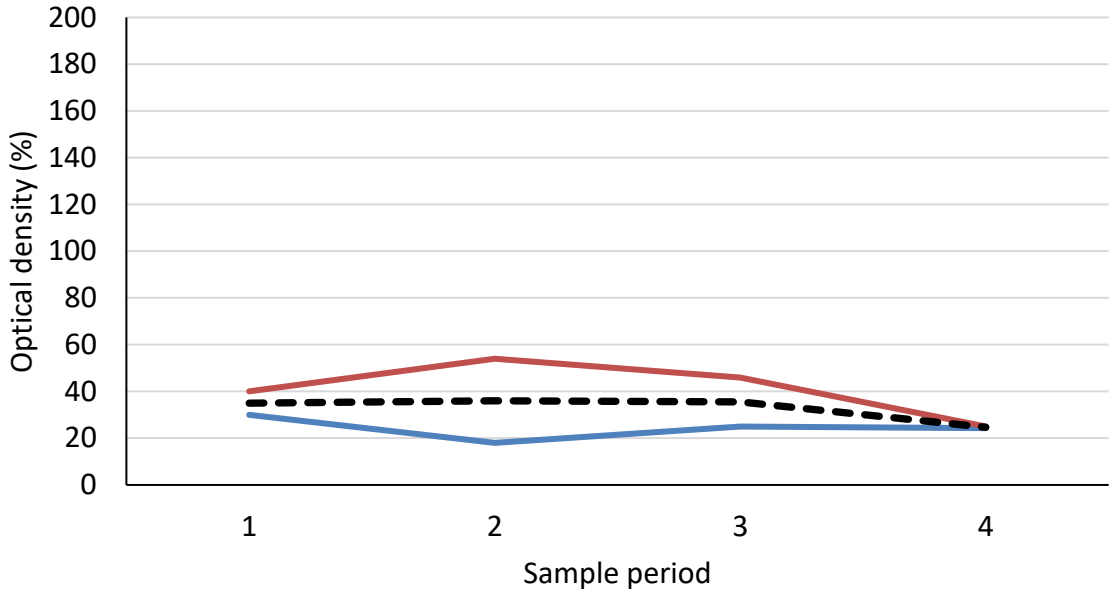


Figure 5-9: ELISA OD% results for the two herds that sero-reverted. Mean OD at each sampling point is represented by the black dotted line.

In the 'other' category there were 18 farms (Figure 5-10). A number of farms in this category hovered around the cut-off value of 30% and others had more 'dramatic' changes between positive and negative. In these herds, they did not strictly seroconvert or sero-revert, and instead the results alternated between positive and negative. Interpretation of the OD trends for these herds should be on an individual basis as the fluctuation in results were different for each herd. The minimum OD value in this category was 5% and the maximum was 199%.

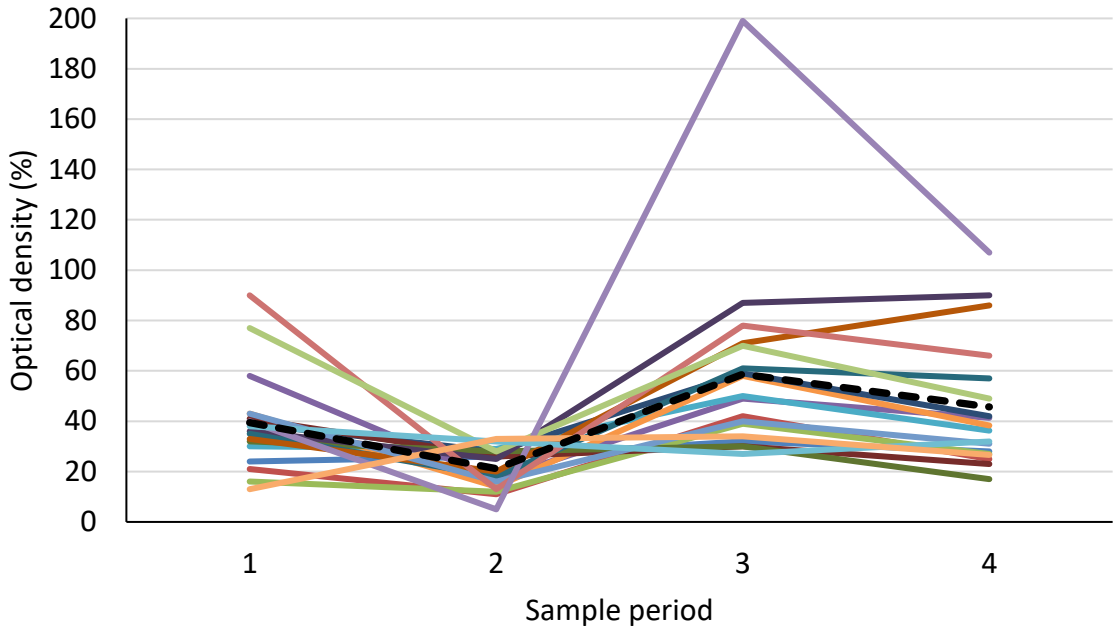


Figure 5-10: ELISA OD% results for all eighteen herds in the 'other' category. Mean OD at each sampling point is represented by the black dotted line.

Three categories were defined as consistently positive, consistently negative, and transitional, with the latter comprising of herds that were initially categorised as seroconverting, sero-reverting and 'other', Table 5-2. In total there were 32 herds in the transitional category. A full summary of each category is shown in Appendix 7 Table 7-1.

Table 5-2: Final categorisation of herds based on the ELISA test OD% cut-off

Category	Definition	No. farms (%)
Consistently positive	Positive in all samples	81 (61)
Consistently negative	Negative in all samples	20 (15)
Transitional	Herds that go between positive and negative	32 (24)
	Total	133

The optical density trends of the transitional category are shown in Figure 5-11.

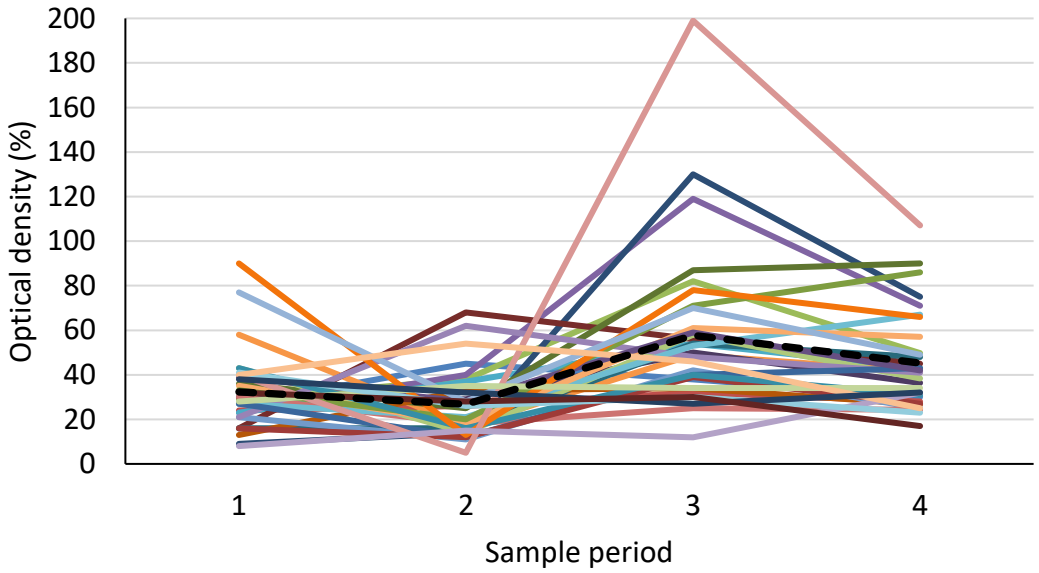


Figure 5-11: ELISA OD% results for all thirty-two herds in the transitional category. Mean OD at each sampling point is represented by the black dotted line.

The mean optical density trends for each of the final three categories are shown in Figure 5-12.

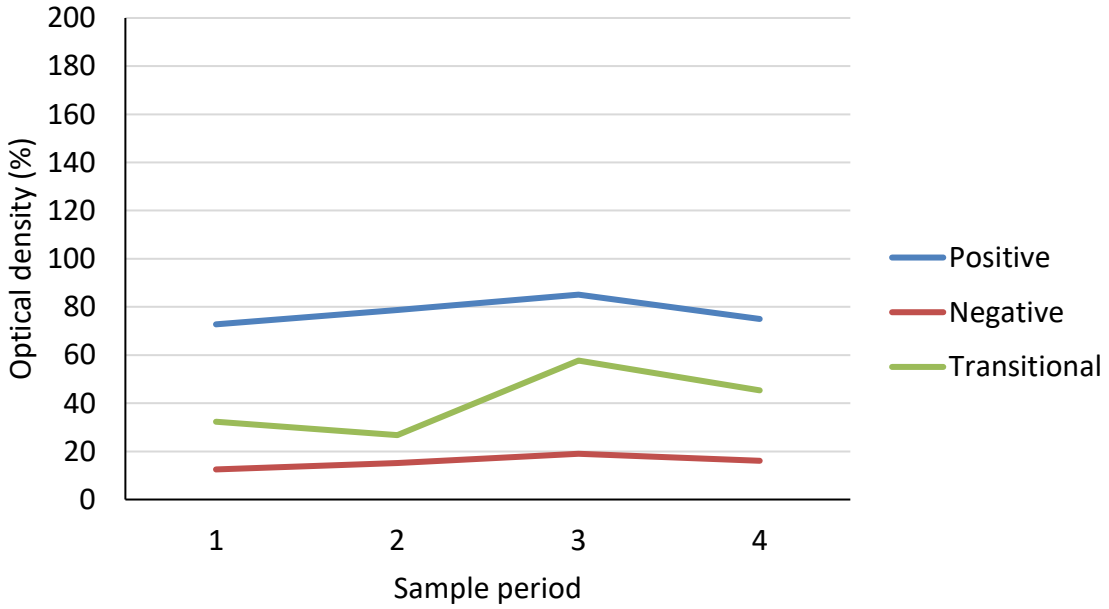


Figure 5-12: Mean OD% results for the three main categories: consistently positive, consistently negative, and transitional

Categorising by degree of change

Most herds (n=54) had 10% or less change in OD value from sample one to sample four, 33 herds decreased by more than 10% from sample one to four, and 46 herds increased by more than 10% from sample one to four (Figure 5-13).

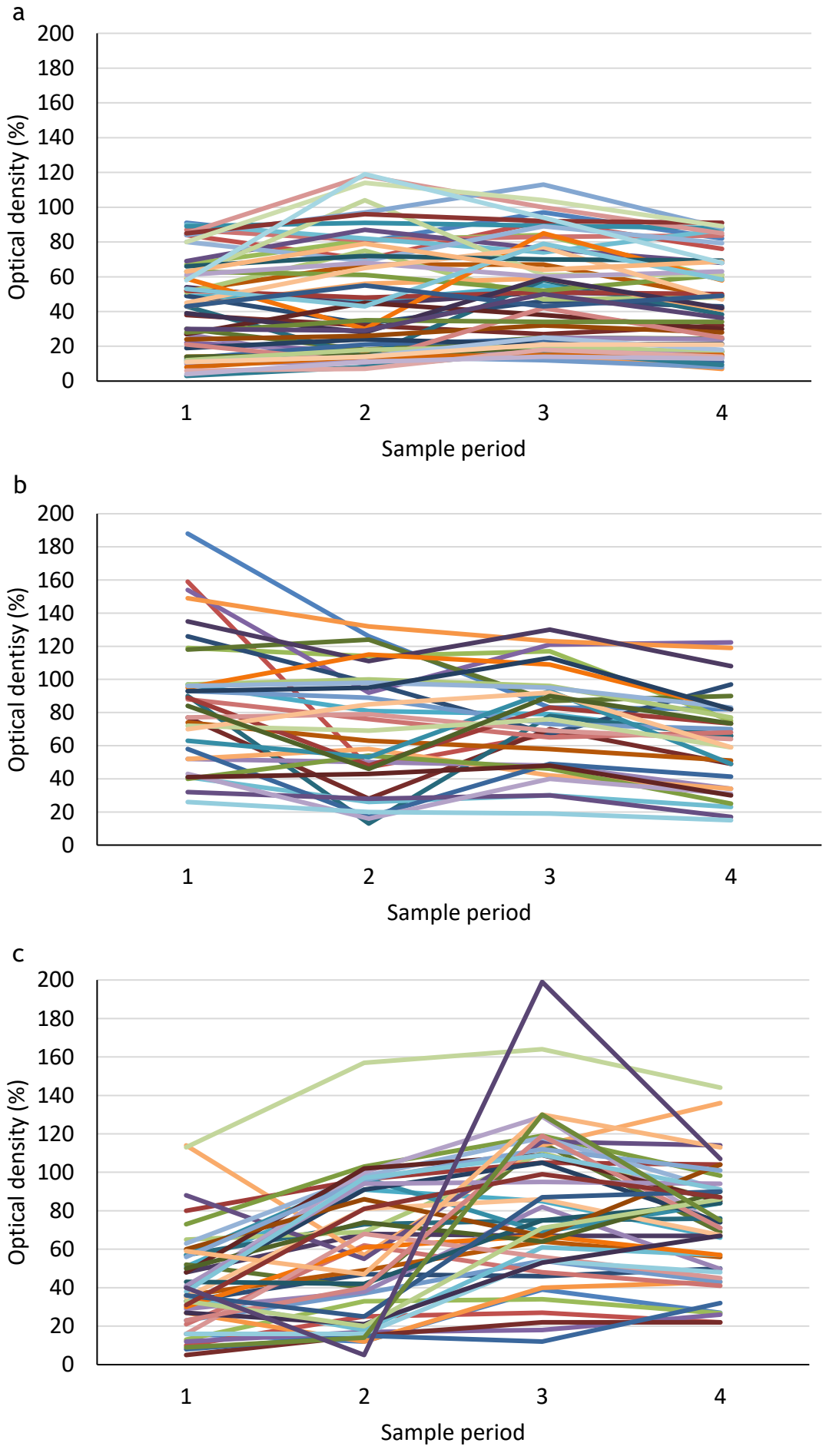


Figure 5-13: Herds that had a change in OD of (a) less than 10%, (b) decrease of more than 10%, (c) increase of more than 10%, from sample one to sample four

Categorising by k-means clustering

The C-H criterion determined that the optimal number of clusters was 7, Figure 5-14.

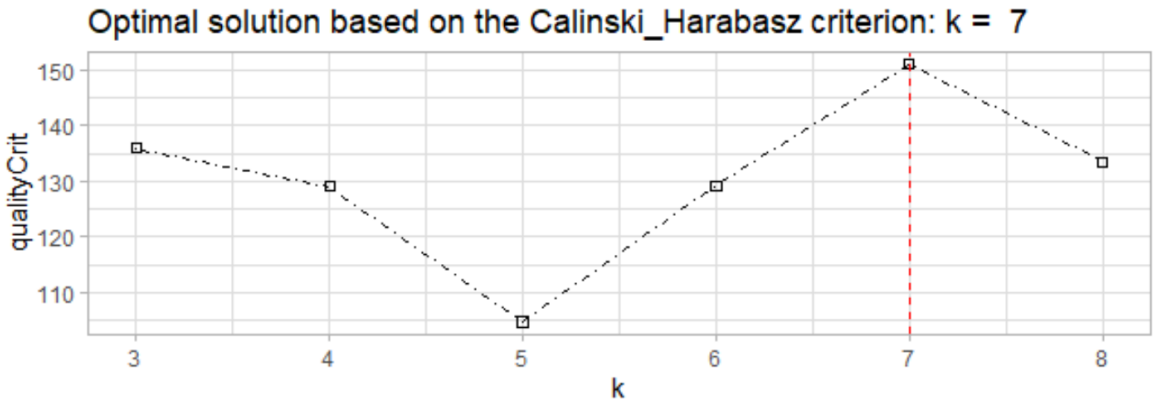


Figure 5-14: Figure showing the optimal value of k based on the Calinski-Harabasz criterion

Upon observation of the clusters, it was evident that there was a considerable amount of overlap between the clusters as shown in the mean OD% trends for each cluster, Figure 5-15.

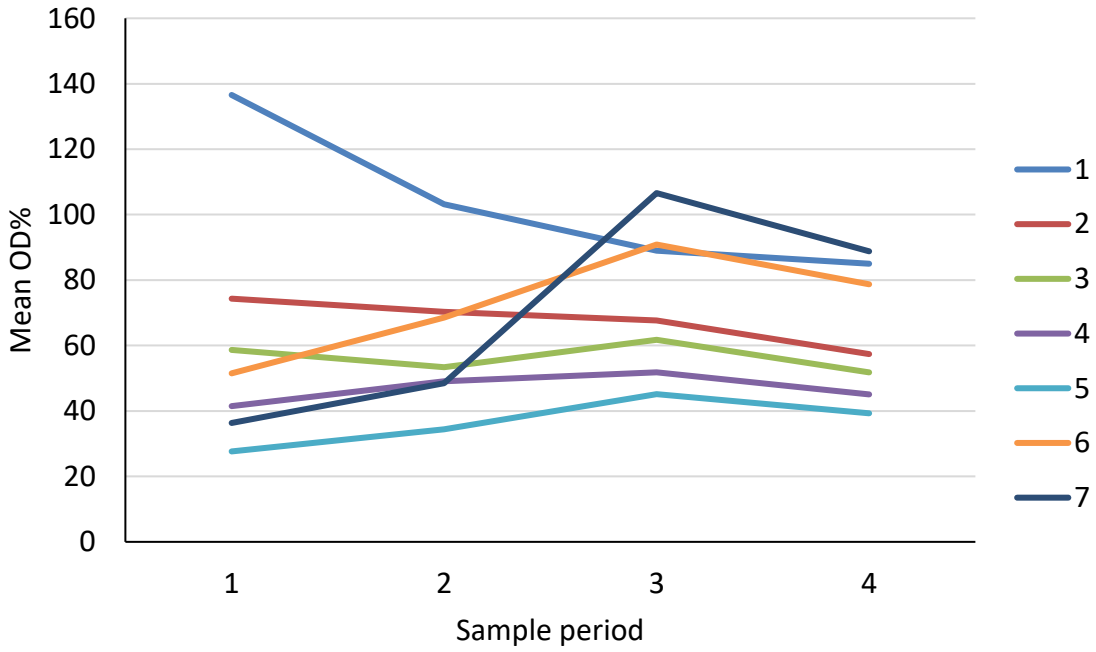


Figure 5-15: Mean OD% for each of the seven clusters created using k-means clustering

Based on epidemiological and biological plausibility, and the fact that the k-value estimate indicated that three clusters was the next optimal number of clusters (k=135.99), Table 5-3, herds were subsequently clustered into three groups.

Table 5-3: Calculated k-mean for three to seven clusters

Number of clusters	k-mean
3	135.99
4	129.07
5	104.71
6	129.23
7	151.01
8	133.42

The mean OD trend for each of the three final clusters is shown in Figure 5-16. On average, Cluster A contained herds that had a general decreasing trend. The mean OD trend for herds in Cluster B was a slight increase, and Cluster C contained herds that increased in OD value over time.

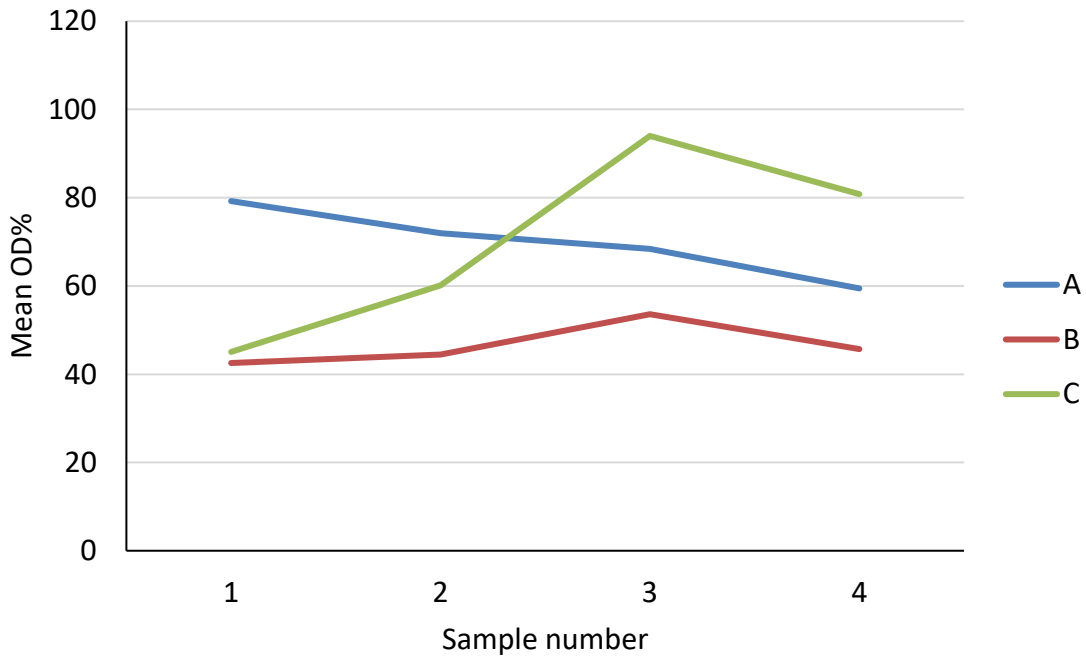


Figure 5-16: Mean OD% for the three clusters identified using k-means clustering

The descriptive statistics for the three clusters are shown in Table 5-4. The majority of herds were grouped into Cluster B (44.4%), 29.3% of herds were in Cluster A, and 26.3% of herds were in Cluster C.

Table 5-4: Descriptive statistics for the three clusters created using k-means clustering

Cluster	Number of herds in cluster	Percentage of herds in cluster	Change	% Change
A	39	29.3	-13.5	-45.6
B	59	44.4	-0.6	-1.7
C	35	26.3	14.1	39.1

In Cluster A, 0% of herds had a positive OD trajectory and 100% had a negative trajectory, whereas in Cluster C, 100% of herds had a positive trajectory and 0% negative. Cluster B had a combination of herds with positive or negative OD trajectories, 61% and 39%, respectively.

The OD trends for all herds over their four sampling points in the three different clusters is shown in Figure 5-17.

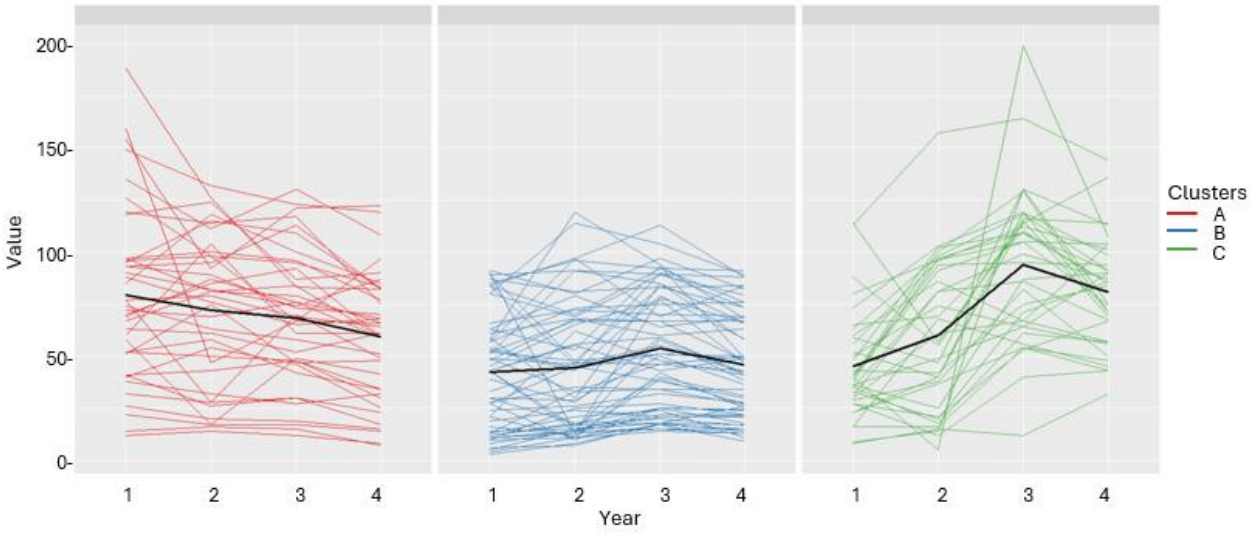


Figure 5-17: OD% trends for all herds in each of the three clusters created using k-means clustering. The mean OD% of each cluster is represented by the black line.

The difference in OD value from sample one to sample four for every herd in each cluster is shown in Figure 5-18.

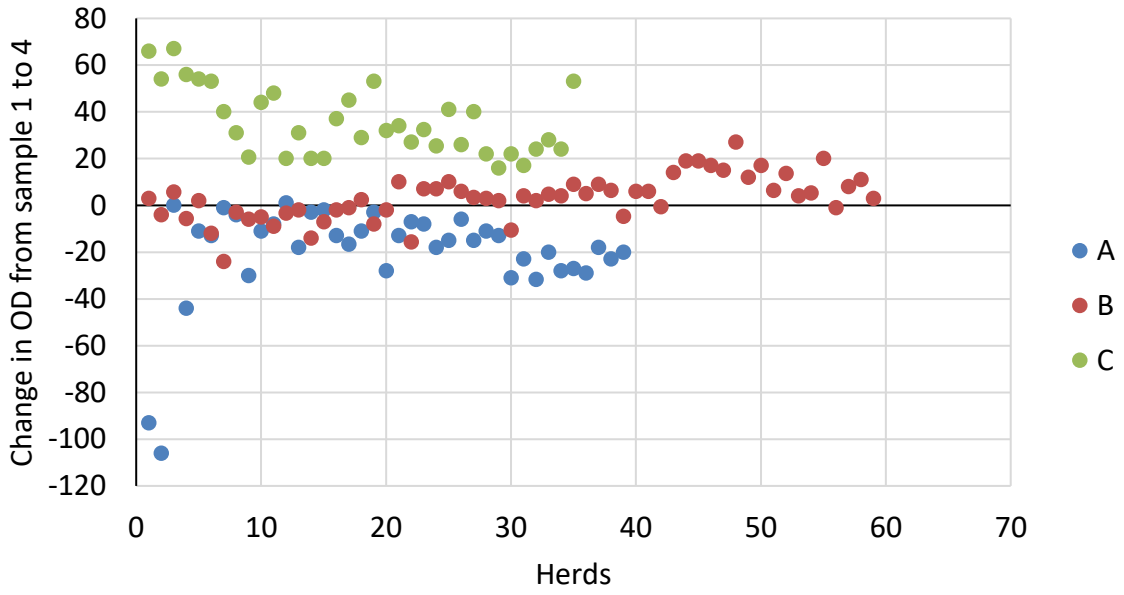


Figure 5-18: Difference in OD% from sample one to four for each herd in the three different clusters using k-means clustering

Agreement between clustering and manual categorisation

The number of herds categorised by clustering with *akclustr* and grouping by the percent of change from sample one to four were compared, Table 5-5.

Table 5-5: Agreement between categorisation using k-means clustering and the degree of change in OD% from sample one to four

Grouping by percent of change from sample 1 to 4	3 clusters using *akclustr*			
	A	B	C	Total
Decreasing	28	11	0	33
Change within 10%	5	43	11	54
Increasing	0	0	35	46
Total	39	59	35	133

The level of agreement between the *akclustr* and manual categorisation was calculated using the following equation:

$$\frac{28 + 43 + 35}{33 + 54 + 46} \times 100$$

There was an accuracy of 80%, or a difference in classification of 20%.

5.3.4 Association between k-means clusters and questionnaire responses

Twenty variables were compared to the k-means cluster assignment, as shown in Appendix 7, Table 7-2. Statistically significant associations, i.e. where $p<0.05$, were identified in the presence of a breeding bull and buying in cattle (0-12 month-olds, 12-24 month-olds, and any cattle).

5.4 Discussion

The aim of this study was to explore methods of categorising herds based on *M. bovis* disease dynamics. To the author's knowledge, this is the first study to observe the longitudinal BTM prevalence of *M. bovis* in dairy herds in Scotland.

5.4.1 Methods of categorising farms

To the author's knowledge, this is the first study to investigate methods of classifying herds based on their *M. bovis* disease status.

Classifying herds based on the result of a single BTM sample may not be as reliable due to the dynamic nature of *M. bovis* antibody responses. Screening herds multiple times per year creates the opportunity to observe disease trends. Identifying consistently positive herds and those where herd-level exposure appears to be increasing could help farmers and vets to develop on-farm control strategies (Sergeant et al., 2019). Sampling of individual animals is labour intensive and can be costly to the farmer. BTM sampling provides a quicker, cheaper alternative to capture the overall picture of the disease status of the lactating herd. Additionally, one BTM sample can be tested for multiple pathogens at the same time, which would be a cost-effective method of screening herds for various diseases. The limitation with sampling BTM is that it only encompasses the lactating herd and does not include dry or sick cows.

Grouping farms based on the binary test result of positive or negative allows for farmers and vets to determine whether the disease level is remaining constant in the herd or fluctuating. Whereas observation of the optical densities offers a more comprehensive look into the actual disease trends at herd-level. A herd may be categorised as consistently negative when categorising based on a positive or negative test result, but upon observation of the OD trends, the BTM

OD may be increasing and heading closer to becoming positive. In these herds it would be beneficial to look at methods of controlling the disease in individual animals before it becomes widespread enough that the BTM is positive.

Categorising based on ELISA test optical density cut-off

Consistently positive:

Within the 81 consistently positive herds, the optical density values ranged between 30% and 199%. Consistently positive herds were categorised as 'low' or 'high' positive based on the herd's mean optical density value. It could be speculated that 'low' positive herds had lower levels of individual cows that were recently exposed to *M. bovis* within the milking herd compared to the 'high' positive herds. Irrespective of whether positive herds were categorised as a 'low' or 'high', *M. bovis* was likely endemic in these herds which led to the continuous, and steady, shedding of antibodies at detectable levels in their BTM.

Little is known about the length of time antibodies are shed after exposure, therefore herds that have been exposed to *M. bovis* may test negative if samples are collected well after active infection has subsided. Byrne et al. (2000) reported that antibody responses remained high in milk from cows with naturally occurring *M. bovis*-associated mastitis at 20 weeks following infection. Antibodies should be detectable in the BTM as long as there are positive individual cows contributing to the BTM. However, there is the dilution effect with milk from the rest of the herd, therefore a sufficient number of cows would need to be actively shedding antibodies to enable the detection of antibodies in the BTM.

Similarly, research to date has not yet determined what proportion of cows contributing to the BTM must be infected to obtain a positive result. This knowledge would benefit veterinary medicine and enable vets to better understand the disease status of a herd upon the collection of a positive BTM sample.

It is not known what proportion of cows in the positive herds would have been clinically infected with *M. bovis*, and this was beyond the scope of the present study. Petersen et al. (2018a) identified that individual cows with clinical

mastitis had higher antibody levels against *M. bovis* in milk than those that had no clinical disease. At herd-level, by sampling from the BTM, the relationship may still be apparent, with higher antibody levels detected in BTM when there is a higher prevalence of clinical mastitis. Although, as previously discussed, this will depend on the number of recently exposed cows that are contributing to the bulk tank. If there are only a handful of clinically infected, antibody-shedding cows contributing to the bulk tank then the sample may be negative.

Furthermore, infections with *M. bovis* can be asymptomatic, while still having an effect on performance, such as reduced milk production (Pothmann et al., 2015). It may be interesting to compare production measurements such as BTM milk fat, protein, urea and conductivity to herds that remained consistently positive in their BTM. Somatic cell count was the only production-related measurement that was collected with each sample.

A larger proportion of herds in the present study were categorised as consistently positive based on the ELISA test cut-off compared to consistently negative herds, suggesting that *M. bovis* may be endemic in Scotland.

Consistently negative:

Antibodies were below the cut-off value of 30% for the entire duration of the study in only 20 herds. This doesn't necessarily imply that there is no *M. bovis* disease present in these herds as there may not have been enough antibody-positive cows contributing to the BTM at the time of sampling. Though it would suggest that these herds are not experiencing active infections at high levels within the herd as antibodies would have been detected at least once.

The first instinct is to recommend that with negative herds appearing to be rarer, that these should be protected by implementing good biosecurity measures such as operating a closed-herd policy. On the other hand, being a negative herd may be considered to be a hinderance as the herd has no immunity within the herd and if *M. bovis* was introduced it could spread rapidly and have significant economic implications to the herd. With an apparent high prevalence of *M. bovis*-positive herds, the likelihood of naïve herds being exposed to *M. bovis* is high.

Transitional:

Farms were categorised as 'transitional' if they did not remain either positive or negative for the duration of the study. This category comprised of herds with a variety of disease trends; seroconverting, sero-reverting, and some with changes back and forth between positive and negative.

In the small number of herds that appeared to hover around the test cut-off of 30%, it was questionable as to whether these herds were in fact consistently positive or negative.

In a diagnostic test with a continuous result, such as the ID Screen® *Mycoplasma bovis* indirect ELISA test used in this study, every value has the potential to be the cut-off point (Habibzadeh et al., 2016). The chosen cut-off value determines the sensitivity and specificity of the test, where the sensitivity is the ability of a test to correctly identify individuals with a disease, and the specificity is the ability to correctly identify individuals without a disease (Torrence, 1997). Both values cannot be increased concurrently. Increasing the cut-off will create a more specific test and reduce the sensitivity, whereas decreasing the cut-off increases the sensitivity and decreases the specificity (Dohoo et al., 2014).

The sensitivity and specificity are not affected by the prevalence of disease in a population. However, the predictive value of the test, which is the ability of a test to determine the absence or presence of the disease in a population, is affected by the disease prevalence (Torrence, 1997). The positive predictive value (PPV) of the test is the probability that an individual with a positive test is truly diseased, and the NPV is the probability that an individual with a negative test result is truly healthy, or non-diseased (Dohoo et al., 2014). When a disease is more prevalent, diagnostic tests are better at detecting true disease rather than ruling it out (Bartol, 2015). Therein, the PPV is higher when the prevalence is higher, and the NPV is reduced. With this in mind, the BTM prevalence of *M. bovis* in Scotland appears to be high, therefore the PPV of the ELISA test used in this study was likely high for interpretation of the results for individual herds.

As well as the effect of disease prevalence, the sensitivity and specificity of the test also influences the PPV and NPV (Thrusfield et al., 2018). When using a more sensitive test, the chance of false negatives is reduced, and thus the NPV is increased. Likewise, higher specificity reduces the chance of false positives,

increasing the PPV. According to the manufacturer, the ELISA test has both a high specificity (100%) and a high sensitivity (95.7%), though, these may be slightly lower (Andersson et al., 2019). Therefore, the likelihood of detecting false positives and negatives in the BTM was lower.

Determining the most appropriate test cut-off point is dependent on the disease in question and the diagnostic strategy. The cut-off point of a diagnostic test is selected to optimise both the test sensitivity and specificity as it is inevitable that the distribution of the measurable substance in diseased and healthy individuals/samples will overlap. Whether it is more important to minimise false positives or false negatives will influence the selection of the test cut-off (Dohoo et al., 2014).

Considering all of the above, when on repeated BTM samples a herd is hovering around the test cut-off, it is important to get further testing of individual cows to understand the disease situation within the herd.

A few herds had particularly interesting disease status trends with much more dramatic changes between positive and negative during the present study. In these herds the underlying reason could be explained by the fact that these samples were taken months apart and would likely not contain milk from the exact same cows at each sampling point. There may also have been changes to management practices that were not captured in the present study that would have explained the changes. Management changes that could reduce the level of disease in a herd include improved hygiene practices in the milking parlour, whereas changes such as buying in new subclinically infected cattle would favour the spread of disease which could result in a higher BTM prevalence. Where herds had all low positive or negative results and one high positive result in the middle of the study, it is possible that one or some of those could have been a false positive.

Herds that seroconverted from negative to positive had likely been newly exposed to *M. bovis* which would cause the antibody levels to steadily increase throughout the study period. Conversely, in herds that sero-reverted from positive to negative, the farmer may have made management changes upon receiving a positive BTM result to minimise disease spread. The level of disease may have also been naturally decreasing as it was self-limiting.

The calving pattern will influence the OD% trend throughout the year. If a herd block calves, there will be large influxes of heifers and cows coming into the lactating herd at specific periods in the year, whereas in a year-round calving system, there will be a constant 'trickle' of animals entering the lactating herd throughout the year. The stress of calving can lead to clinical or subclinical mastitis (*M. bovis*-associated or caused by other pathogens) (Nicholas et al., 2016). If there is a larger influx of newly calved animals entering the lactating herd at one time, such as in block calving herds, this can increase the BTM OD% around that time or in the following weeks. Parker et al. (2017b) found an association between the BTM OD% and the length of time since the calving period commenced. In herds operating on a seasonal or block calving pattern, the BTM OD% was higher between five and eight weeks after the start of the calving period. However, the mean BTM OD% was higher in year-round calving herds, which the authors attributed to the fact that there is a continuous influx of cows entering the lactating herd throughout the year. Likewise, in block calving herds there will be larger proportions of dry cows at specific timepoints whereas in year-round calving herds there will be smaller proportions of dry cows throughout the year. When there is a greater proportion of dry cows at once, the OD% may increase if the cows still contributing are positive as there is less dilution. Alternatively, if a number of dry cows were antibody positive, the OD% could decrease.

Other factors such as the proportion of heifers entering the lactating herd and the proportion of sick cows may affect the BTM OD%. As discussed in Chapter 4, if there are cows with clinical mastitis and/or receiving antibiotic treatment, they should not be contributing to the BTM. Once they have clinically recovered or have surpassed the withdrawal period of the antibiotics and re-enter the lactating herd, they may still be producing antibodies which could increase the BTM OD%.

The proportion of first-lactating heifers entering the BTM may also affect the BTM OD%. These animals are at a higher risk of clinical mastitis due to calving stress. Furthermore, they may also be naïve to endemic diseases circulating in the milking herd as they are often managed separately until calving. This can lead to a spike in mastitis if there are a higher number of first-lactating heifers entering at the same time.

In summary, the time of year in which samples are collected may influence the BTM OD%, and this will vary within different herds depending on different management practices such as the calving pattern. Further research is required into the dynamics of *M. bovis* antibodies in the BTM.

For herds that were categorised as 'transitional' it may be of interest to observe these herds closely to determine what was occurring in these herds on an individual basis, particularly for the herds that appear to fluctuate between positive and negative.

Percentage change in OD and k-means clustering

Percentage change in OD:

Looking at the degree of change between samples one and four offers more insight into the disease trends within the herd. Where a herd may repeatedly test positive in their BTM samples, the actual OD% may be changing or remaining relatively stable.

A large proportion of herds had a 10% or less change in OD value from sample one to four. This category can be segregated into two subgroups, positive herds wherein the OD levels remained stable, and negative herds with little change in the OD levels. Herds that remained at a stable negative BTM OD% throughout the study likely did not have any active *M. bovis* infection present nor had they been recently exposed. *M. bovis* is likely to be endemic in the herds that remained positive at a stable rate. This suggests that once *M. bovis* is introduced into a herd and is well established that it can remain at a relatively constant level. It is not known if the stable positive BTM results in these herds is reflected in the appearance of clinical disease and/or subclinical effects on performance.

In the 46 herds with an increase in OD% of more than 10%, it can be assumed that the level of infection was increasing within the lactating herd. A proportion of herds in this category seroconverted from negative to positive, likely due to the recent introduction of *M. bovis*. In these herds that were already positive in their first BTM sample, it is possible that within-herd hygiene measures were not robust enough to prevent the spread of disease, which led to the continuous increase in OD% that was observed.

In the 33 herds with a decreasing OD% of greater than 10%, this could suggest that the level of *M. bovis* infection is reducing and there has been little or no recent infection within the herd.

Model-based clustering:

The use of computational clustering methods is a relatively novel field in veterinary epidemiology, particularly for classifying longitudinal datasets. Only a handful of recent studies have proven its use in the veterinary sciences, particularly for identifying patterns and groups of farms based on results of their BTM samples.

One of the limitations of k-means clustering is that outliers are not represented by cluster centroids (Gan and Ng, 2017). As shown in the present study, there were outliers in the data. The inertia of the clusters could have been improved by removing the outliers, which would thus increase the reliability of the clusters. Although, the outliers didn't appear to have too much of an impact on cluster membership as upon observation of the clusters, herds with similar BTM OD trajectories were clustered together.

K-means clustering was the chosen method used in the present study due to its ease of use and the researcher's limited experience in computational clustering. Also, k-means clustering does not require the data to be normally distributed. When using k-means clustering, the number of clusters (k) must be predefined prior to running the algorithm. In the present study, the C-H index was computed. For all clusters, the ratio of the sum of inter-cluster dispersion and the sum of the intra-cluster dispersion is calculated. When the clusters are denser within and well separated from each other, the C-H index will be higher. The higher the C-H index, the better the clustering. Alternatively, approaches such as the elbow method of silhouette method could have been used. The C-H index was used as it is the default approach in the *akclustr* package in R (Adepeju et al., 2021).

Computational clustering methods are beneficial to the industry if the clusters identified are biologically plausible. In the present study, the C-H index determined that the optimal number of clusters for the dataset was seven. On observation of the original seven clusters, it was apparent that there was a great

deal of variation in the rate of improvement or depreciation on different farms. However, as mentioned previously, there is no real benefit of having seven different clusters of farms based on their BTM OD disease trajectory, which is why it was decided to go with the second highest C-H index of three clusters.

In the present study, not all herds with missing values were discarded. The mean OD% was calculated for herds that had submitted three BTM samples and this value was used to create a fourth BTM result. Typically, in cluster analysis, trajectories that contain missing values are excluded from the data (Genolini and Falissard, 2010). The downside of this is that datasets are then reduced in size which can have an impact on the quality of the clustering (de Souto et al., 2015). Rather than including the total 181 herds in the cluster analysis, only 133 herds fit the criteria of having three or four BTM results.

Missing values within a longitudinal dataset may be a characteristic of a specific cluster, for example those that drop out early from a study (Genolini and Falissard, 2010). Within the dataset from the present study, this may not have been evident due to the smaller sample size and limited number of sampling points. This type of observation is more apparent in longitudinal studies that collect larger numbers of repeated samples, for example, in clinical studies with daily sampling points over the period of one week or longer.

In the present study, it was speculated that the herds that dropped out after the first or second sampling point may have done so as they were satisfied upon receiving one or two BTM results. In Chapter 3, it was reported that there was no association between the previous BTM result and whether herds dropped out or continued to participate in the study.

Comparison and summaries of different methods:

Categorising farms based on the rate of change between the first and fourth sample offers a benefit that cannot be provided by categorising herds based on the test cut-off. Farms may remain consistently positive but in actual fact have a decreasing trend towards becoming classified as negative. Similarly, a herd may consistently test negative while the OD is in fact increasing towards the test cut-off.

Using model-based clustering methods requires a level of understanding of statistics, therefore may not be of direct use to farmers and some veterinary practitioners. These methods don't strictly distinguish whether a herd is testing positive or negative, however, they do provide an insight into the general trend of disease over time. When categorised by the degree of change, the majority of herds had a change of 10% or less in the optical density value between samples one and four. Similarly, there was a higher number of herds in k-means cluster B which contained herds that on average had a small change in their OD slope.

This stability in BTM OD% suggests that in these herds there was an equal number of cows entering and leaving the lactating group at the same time. The cattle that should be contributing to the BTM, or that are eligible to contribute, can be grouped into: apparently healthy cows with no clinical symptoms, sick cows which may or may not be receiving treatment, dry cows, cull cows, in-calf heifers, and newly purchased cows/heifers (Figure 5-19). In each of these groups, there could be a mixture of *M. bovis* positive and *M. bovis* negative cows, or it could be that some of the groups are all/mostly positive and others negative, which could result in generally stable BTM OD results throughout the year. The high number of study herds with stable OD trajectories may imply that once *M. bovis* is present within a herd it remains endemic.

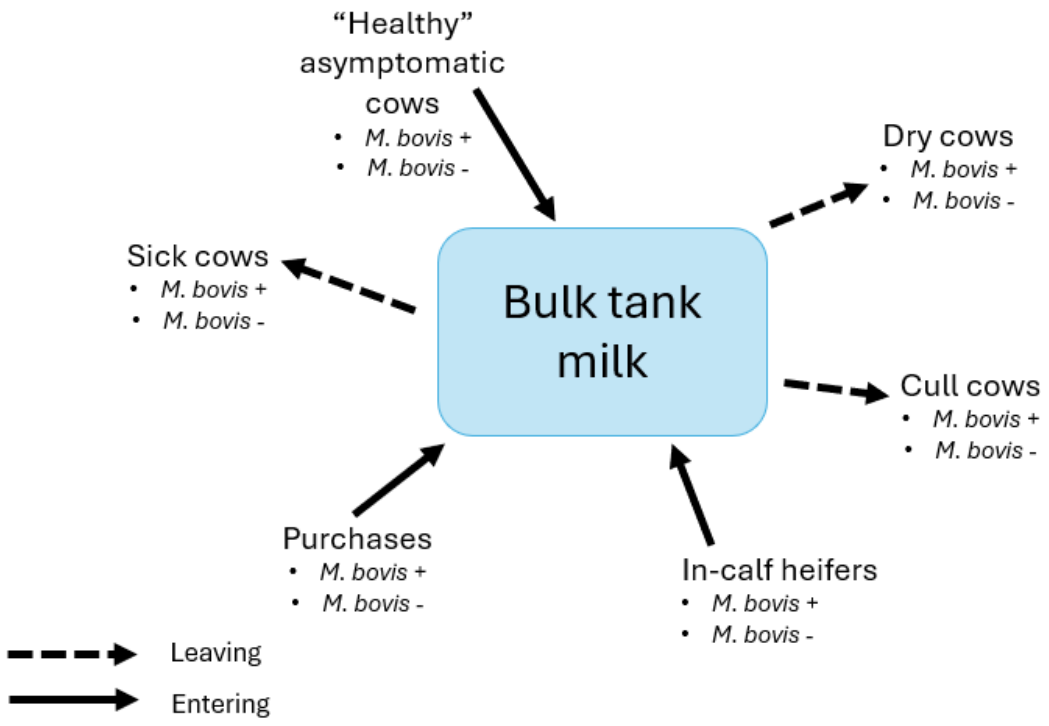


Figure 5-19: Figure illustrating the groups of cows that contribute to the BTM.

The percentage of agreement between the k-means clustering and the manual method of categorising by degree of change was 80%. Both approaches are essentially calculating a similar value. For the k-means clustering, the rate of change/steepness of the slope was calculated, and for the manual method, this was calculated as sample 4 OD minus sample 1 OD.

This study has demonstrated that despite the fact that cluster methods are exploratory and do not describe the quality of inertia of the clusters, the clusters generated within the data were meaningful, i.e. correct grouping of increasing, decreasing and stable OD trendlines.

Rather than categorising herds by a change of 10% or more, this could have been increased to 15% or 20%. It is not known how significant a change in OD of 10%, for example from 35% to 45%, compared to a change of 35% to 55%. This comes back to one of the major gaps in our knowledge of *M. bovis* which is how many positive cows need to be contributing to the BTM at the time of sampling to give a positive result. Furthermore, it is not known what the difference is in the proportion of positive cows contributing to the BTM to produce an OD of 40% compared to 75%. A change in OD% of between 10% and 20% may be of significance, or it may be more of an issue when there is a change of 30% or more.

If repeated BTM samples are collected from a herd and tested for the presence of a specific disease, or antibodies, simply eye-balling the changes in the results is a good method of determining whether the level of disease is increasing, decreasing or at a steady level. It is also useful to both observe the change in OD% overtime and record whether the results lie above or below the test cut-off.

How classification of herds based on BTM could be utilised

Testing of BTM is a quick and easy method for farmers to capture the disease status of their herd. One sample can be tested for multiple pathogens, increasing disease control efficiency. Exclusively testing BTM samples only provides a snapshot of the lactating herd that contributed to the BTM at the time of sampling. Therefore, the result only represents the group of sampled animals, and does not include youngstock, dry cows or the sick pen. This is also why repeated BTM sampling is highly effective at monitoring disease trends

within a herd. Observing trends and changes in BTM over time can give an indication of herd status; free from disease, recovering from disease, endemically infected, or newly infected.

Additionally, identifying factors that are associated with the disease trends over time would enable those within the dairy industry to make informed decisions about their herd management and what impacts they may have on their herd in relation to *M. bovis*. In this Chapter, associations between the questionnaire responses and the k-means cluster assignment of herds were briefly assessed by univariable analysis, though this was not taken further to a multinomial analysis. In this study, the questionnaire was answered by farmers at the first sampling point and the outcome variable (cluster assignment) reflects the results over a one-year period. The cluster assignment reflects a dynamic process whereas most of the questions (except Q4) were 'static' or rather true of that specific timepoint but may have changed over the course of the study. Where these associations may be made, there is a risk of misclassification bias, and consequently over- or under-estimations of possible associations can be made.

When the questionnaire was designed, a causal web was created (Figure 4-1 in Chapter 4) to identify specific factors that may be associated with the presence or absence of *M. bovis* and *M. bovis* antibodies, not the change in the antibody/OD% levels over time. Some of the possible associations shown in Table A7-4 in Appendix 7 make biological sense, such as buying in cattle. Herds that had an increasing trend were more likely to have bought in cattle than not. This makes biological sense as buying in cattle increases the risk of introducing the disease, therefore the OD trend would increase over time. Also, the introduction of naïve animals into an infected herd could become infected and thus there would be an increase in antibodies produced within the herd.

To correctly identify factors that were associated with increasing, decreasing or stable OD trends, it would have been better to ask a retrospective questionnaire at the end of the study at the fourth sampling point, or at each sampling point. Furthermore, different questions could have been asked, where potential associations could be better understood. Computational methods of clustering do not statistically test the certainty of cluster existence and are purely investigative (Genolini and Falissard, 2010). However, with a simple dataset such

as the dataset in the present study, it is relatively easy to determine if the clusters created are comparable.

National control and surveillance schemes are beneficial to the agricultural industry as they improve financial output and overall herd performance. There is currently no national control programme for *M. bovis* in Scotland. The development of a surveillance strategy such as the national *M. bovis* eradication scheme in NZ requires information on the prevalence of *M. bovis*. If the prevalence of *M. bovis* is believed to be very low and is having little impact on herds, it could be argued that there would be no requirement for a national control scheme. Conversely, if there is evidence that *M. bovis* is highly prevalent in Scotland causing clinical disease and resulting in financial losses, there may be an industry-driven push for a national monitoring and control strategy.

The formulation of a national control strategy for a disease such as *M. bovis* requires collaboration between the dairy industry, veterinary practitioners, and the government to identify the best approach. Initially, screening schemes for *M. bovis* would likely be voluntary rather than mandatory, unless there was any evidence to suggest that the disease had a high mortality rate and was spreading rapidly. This may include, though not be limited to, BTM sampling, individual cow milk sampling and could incorporate youngstock screening. The sampling would also be dependent on the overall aims of the national strategy, i.e. different numbers of animals may need to be sampled if the aim is to prove disease freedom rather than identifying infected individuals or herds.

If national surveillance of *M. bovis* was established in Scotland, it may follow the same structure as that of the BVD eradication scheme (Shortall, 2022) where there are multiple phases/stages that the national dairy industry collectively moves through.

The future of *M. bovis* testing, and the initiation of potential control and monitoring schemes will be discussed in further detail in Chapter 7.

5.4.2 Conclusions

It is not known if the proportion of herds within each of the categories based on BTM OD trajectories reflects the patterns in the total dairy herd population in

Scotland. When applying the k-means clustering and degree of change categorising, there was a large proportion of herds that had a more stable BTM OD trajectory. This could suggest that once *M. bovis* is established in a herd it remains at a fairly stable level. Unlike many other countries, the primary concern for *M. bovis* within a herd is BRD in youngstock rather than mastitis. The findings of this study may explain the reason for mycoplasma-mastitis being of less importance if (a) the disease level remains stable and (b) if there is an apparent low incidence of clinical mycoplasma-mastitis in herds. These results highlight the importance of continuing research into *M. bovis* in Scotland, particularly to address herd-level impacts and associations between positivity and the occurrence of clinical mastitis.

There are various methods that can be applied to categorise farms based on BTM prevalence. The choice of method used will depend on the question to be answered. It is also important to consider the underlying biological reason for categorising farms, whether the aim is to identify herds that appear to be consistently challenged at various levels of disease, or to quantify the degree of change in OD to determine whether the disease status is remaining constant or if a farm has a newly introduced disease challenge.

Although categorising based on the diagnostic test cut-off appears to be an effective approach, observation of the OD trend may be more beneficial to farmers and vets as this method can be used to predict whether the disease is remaining constant, increasing or decreasing.

This was an exploratory piece of work aimed at trialling different methods of classifying herds based on the results of repeated BTM sampling. By classifying herds, farmers and vets can monitor possible disease trends at herd-level and thus improve their treatment regimes.

At the time of writing this thesis, *M. bovis* is not considered as a pathogen of significance in Scotland, hence the lack of national control or monitoring strategies. However, the UK Cattle Expert Group published a document in 2018 which summarised the knowledge gaps that needed to be addressed on *M. bovis* in the UK (UK Cattle Expert Group, 2018). One key area discussed was an estimation of the prevalence of *M. bovis*, which would support the development of disease eradication programmes. The findings of this study may initiate

conversations surrounding the need for *M. bovis* monitoring or eradication programmes in Scotland.

Chapter 6 Seroprevalence of *Mycoplasma bovis* in Scottish calves

6.1 Introduction

6.1.1 Overview of Mycoplasma bovis

Infections with *M. bovis* have significant economic and welfare implications for dairy herds due to increased mortality and morbidity, treatment costs, poor production, and reduces the growth rates in calves (Cernicchiaro et al., 2013; Nicholas et al., 2008). Losses are also attributed to take out undesired early departure of adult cows from the herd by death, euthanasia, or slaughter (Petersen et al., 2019).

M. bovis infections in calves manifest as chronic respiratory disease, arthritis, and otitis media (Foster et al., 2009), all of which are difficult to treat due to most antimicrobial groups being ineffective against the pathogen (Maunsell et al., 2011). Some cattle develop no symptoms and become asymptomatic carriers remaining as a continuous source of infection to the rest of the herd (Punyapornwithaya et al., 2010).

Respiratory disease infections are highly likely to be multifactorial, with multiple pathogens being present, some of which are more prominent than others (Pratelli et al., 2021). *M. bovis* is one of the pathogens involved in the BRDC, containing a range of bacteria, viruses, and other stressors that contribute towards respiratory disease in youngstock (Cirone et al., 2019). Previously, *M. bovis* was considered secondary and opportunistic to other pathogens, requiring stressors such as transportation or infections with other pathogens to initiate disease (Kusiluka et al., 2000). A calf will be challenged with a stressor or viral pathogen, a bacterial infection may follow with additional potential infection with *M bovis*. When sampling from calves with respiratory disease, *M. bovis* may be present in a large number of instances, though may not always be having the greatest impact on the calf.

6.1.2 Transmission and risk factors

M. bovis is introduced to herds through contact with infectious cattle, generally purchased asymptomatic cattle, over the farm boundary or at an agricultural show or market (Punyapornwithaya et al., 2010; Ridley and Hateley, 2018). Once in a herd, the pathogen transmits readily throughout the herd (Calcutt et al., 2018), and within groups of calves (Wawegama et al., 2016). Cows' milk and colostrum were considered to be a major source of infection for youngstock from the lactating herd (Bennett and Jasper, 1977a). The findings of Chapter 4 and 5 suggest otherwise with only four herds testing positive by PCR which tests for the presence of *M. bovis* DNA, and the majority of herds feeding cows' milk to their calves. Other important routes of transmission are via inhalation of infectious aerosols, nose-to-nose contact with infected individuals, and fomites such as feeding equipment and bedding (Maunsell et al., 2011).

The 'gold standard' is to feed cows' colostrum within the first 12 hours of life and switch straight onto artificial milk, however, there can be an abundance of waste milk in a herd, coupled with the cost of milk powder is high, this makes feeding waste milk much more convenient and feasible to farmers (Selim and Cullor, 1997). Furthermore, feeding of waste milk to calves is not permissible to farmers within the Red Tractor Farm Assurance Scheme. Disease transmission in milk and colostrum can be prevented by pasteurisation as this kills *M. bovis* (Butler et al., 2000), however, this is not feasible for all farmers and thus is not standard practice.

As well as direct transmission from cows to calves via milk and colostrum, *M. bovis* may also be spread if the different groups of animals are housed in the same airspace (Nicholas et al., 2002). Additionally, if the same personnel work with the cows and calves they may also be a source of transmission between the two groups.

6.1.3 Treatment and control

Treatment options for *M. bovis* infections are limited due to the fact that most antimicrobial groups are ineffective against the pathogen (UK Cattle Expert Group, 2018). Many of the *M. bovis* control measures are based on optimal

management practices for general disease prevention. Maintaining good on-farm hygiene will minimise the risk of the disease, including *M. bovis*, spreading throughout the herd (Baraitareanu and Vidu, 2021).

As cows' milk and colostrum is a potential source of infection to youngstock, if feasible, milk and colostrum should be pasteurised prior to feeding calves. Furthermore, feeding waste milk and colostrum to calves should be avoided. Regularly cleaning and disinfecting feeding equipment also prevents transmission within the group, especially if calves are either group fed or if buckets are swapped between individuals.

Mixing youngstock of varying ages should be avoided and animals should remain in the same groups beyond weaning to prevent the exposure of younger, naïve calves to older calves. This is not always possible depending on the calving period, size of the herd, and layout of the farm.

Vaccines against *M. bovis*

At the time of conducting this study, a commercial vaccine against *M. bovis* was developed, the Myco-B One-Dose™ (American Animal Health, Grand Prairie, Texas, USA). In 2019, this inactivated vaccine was first imported into the UK under a special import license by a veterinarian in Aberdeen who trialled the vaccine on four of his client's farms (Fowlie, 2021). The effect of using the vaccine on antimicrobial use (AMU) and post-weaning mortality was compared to four farms that did not use the vaccine. Both the post-weaning mortality rate and AMU decreased in herds that vaccinated, however, as the author stated, the results must be interpreted with caution because all calf mortalities recorded were included and not those only with *M. bovis*-associated disease. Also, the study population was very small. Nevertheless, this study encouraged farmers and vets throughout the country to use this vaccine to protect their herd against *M. bovis*.

The Myco-B™ vaccine is a one-shot dose that can be administered to calves as young as 60 days of age and can also be given to dry cows. An issue with this minimum age is that maternal antibodies can persist in calves up to six months of age, though the exact duration for different pathogens is not fully understood (Windeyer and Gamsjäger, 2019). Maternal antibodies are mainly transferred to

calves via passive immunity from colostrum (Lopez and Heinrichs, 2022) and only very minimal quantities are transferred *in utero* (Goddeeris et al., 1998). Over time, the level of maternal antibodies decreases, influenced by variables including the time of receiving colostrum and colostrum quality.

The effectiveness of vaccines in younger calves can be lower as maternal antibodies can interfere with their immune response to the vaccine by neutralising the antigen before the immune system can develop a response (Niewiesk, 2014). The concentration of maternal antibodies in the calf at the time of vaccination will influence the degree of vaccine interference. In younger calves with higher levels of maternal antibodies, there is a greater chance of vaccine neutralisation. This is why it is crucial not to vaccinate calves too young. Another consideration when choosing when to vaccinate calves is the immune gap. This is the period when the maternal antibodies are too low to provide protection, but the antibody titre remains too high for vaccination (Pastoret, 2007). During this time, the calves are susceptible to infectious diseases as they are not protected by a vaccine nor maternal antibodies. As the duration of maternal antibodies is variable, it is not clear when exactly the immune gap will occur in every calf, and ideally, they will be vaccinated once the maternal antibody titres are low and before they are exposed to potential pathogens.

Ultimately, the development of this commercial vaccine was a game-changer for the Scottish dairy industry, providing farmers with the ability to protect their herds from *M. bovis* entering and reducing their reliance on the use of antimicrobials.

6.1.4 Prevalence estimates in youngstock

Due to the fact that respiratory disease in calves is often multifactorial, it is difficult to determine how often *M. bovis* is the primary causal pathogen in these infections. Furthermore, it is notable that the presence of *M. bovis* does not always result in clinical disease in youngstock. Previous studies on beef cattle have reported varying prevalence estimates of *M. bovis* in youngstock. In pens of 8-12 bull calves in a beef fattening operation, the within-pen prevalence of *M. bovis* ranged between 8-100% (Timsit et al., 2012). One hundred percent of veal calves tested positive for the presence of *M. bovis* at slaughter in Northern

Italy, none of which presented with any clinical symptoms indicative of respiratory disease prior to slaughter (Radaelli et al., 2008). In another study, the authors were unable to culture *M. bovis* from any healthy calves, however, the pathogen was cultured on 2 out of 100 visibly healthy lungs from calves at postmortem (Thomas et al., 2002b). More recently, *M. bovis* and antibodies to *M. bovis* were isolated from blood samples taken from 61% of apparently healthy calves in Western Australia (Gogoi-Tiwari et al., 2022).

According to the Great Britain Veterinary Diagnostic Network, the number of pneumonia cases diagnosed with *M. bovis* increased from 16 in 2012 to 68 in 2021 (GB Veterinary Diagnostic Network, 2023), though this data only contains diagnoses from submissions made to the network.

6.1.5 Association between BTM and calf seroprevalence

Very little is known about the association between the seroprevalence of *M. bovis* in adult cows and youngstock. As previously mentioned, there are many potential routes of transmission that exist between the lactating herd and youngstock. Presumably, where the pathogen is endemic in the adult herd, there is a clear risk of transmission to youngstock, especially where within-herd biosecurity is sub-optimal. Associations between pathogen prevalence in BTM and mortality in youngstock has been previously described in *Salmonella Dublin* (Nielsen et al., 2010), and *M. bovis* (Hurri et al., 2022), yet associations with calf seroprevalence are not described.

6.1.6 Study aims

The aims of the present study were to i) identify herds that were free of *M. bovis* disease in their youngstock and ii) determine if there is an association between BTM prevalence and calf seroprevalence of *M. bovis*.

6.2 Methods

6.2.1 Recruitment of participants

Dairy herds that were categorised as consistently positive, consistently negative, and transitional based on BTM results as described previously (Chapter 4) were invited to participate in the follow-up calf study. Farmers were approached between July and October 2021.

Farms were eligible to participate if they had completed three or four sampling points and if they did not use a vaccine against *M. bovis*. Eighty-one farms that met the eligibility criteria expressed their interest in participating. An additional eight farms were unable to participate as they used a vaccine against *M. bovis*.

The aim was to sample from an equal number of farms from each category. The total number of farms that could be sampled from was limited to 60 due to the budget. As there were fewer negative (n=13) and transitional (n=20) herds interested in participating, all of those farms were approached. Forty-eight out of the 81 farms interested in participating were consistently positive in the BTM prevalence study. The mean bulk milk OD value for positive herds was calculated and the herds were listed from smallest to largest. The list was split in half to create a ‘low’ and ‘high’ consistently positive group. In each group farms were randomised in a list and the first 10 farms from each group were selected.

6.2.2 Study design and sample size

A cross-sectional study was designed to investigate the presence of *M. bovis* antibodies in calves. The study took place between November 2021 and July 2022. Participating farms were sent a sampling kit that contained a consent form, participant information sheet, questionnaire, sample submission form, blood sampling tubes, and a bulk milk tube containing a preservative tablet. The participants were asked to blood sample 20 calves with their registered vet, 10 calves aged 4 to 8 months old, and 10 calves aged 10 to 14 months old. A BTM sample was also to be collected and returned along with the completed sample submission form, questionnaire, and signed consent form.

The median number of calves on farms in the target population aged 0-12 months old was 100 according to responses to the questionnaire in the BTM prevalence study (Chapter 4). In a population of 100, to be 99% certain of including at least one positive calf if the disease was present at >50%, 10 calves needed to be sampled (Cannon and Roe, 1982). The prevalence of 50% was chosen due to the highly transmissible nature of the pathogen; in a homogenously mixing group, most calves will either be seronegative or seropositive (C. Mason, Personal Communication). It was decided to sample 10 calves in each of the two age groups to ensure under sampling was avoided.

Ethical approval was obtained from the University of Glasgow's School of Veterinary Medicine Ethics Committee prior to conducting the study.

6.2.3 Sample testing

Both the BTM and calf blood samples were tested at SRUC Veterinary Services Veterinary and Analytical Laboratory, Edinburgh, using the commercially available IDvet *M. bovis* screen indirect ELISA to test for the presence of *M. bovis*-antibodies. According to the manufacturer, the test sensitivity and specificity is 95.7% and 100%, respectively, though the specificity is likely to be 98% (C. Mason, Personal Communication). The ELISA test OD cut-off is 60% for blood samples, where 60% or more is positive and less than 60% is negative. For BTM samples, the OD cut-off was 30%, with 30% or more being positive and less than 30% negative.

6.2.4 Case definitions

The seroprevalence was estimated at calf-level, group-level, and herd-level. At the individual calf-level, a case was defined as a calf that tested antibody positive based on the cut-off. Group level prevalence was defined as the number of antibody positive calves in each of the two age groups. At herd level, a case herd was defined as a herd that contained at least one seropositive calf, based on the fact that within herds most calves are all seropositive or seronegative.

6.2.5 Test for normality

The OD% values for the two age groups were both not normally distributed, therefore an unpaired two-samples Wilcoxon test was performed on the data to determine if there was a difference in OD% between the age groups.

6.2.6 Test for association between BTM and calf seroprevalence

Data from two separate studies were used to test for associations between BTM and calf seroprevalences: longitudinal BTM *M. bovis*-antibody test results (Chapter 4), calf *M. bovis* seroprevalence results, and the BTM sample collected in the calf seroprevalence study.

Longitudinal bulk milk data was collected as described in Chapter 4. Briefly, dairy herds throughout Scotland were recruited to participate in a BTM prevalence study that required participants to submit four BTM samples roughly three months apart between August 2020 and October 2021. The samples were tested at the SRUC Veterinary Services Veterinary and Analytical Laboratory, Edinburgh using the IDvet *M. bovis* screen indirect ELISA to detect the presence of antibodies to *M. bovis*.

Associations between BTM results and calf results were assessed via two methods: firstly, based on the optical densities of both the calf and BTM test results (OD%). The mean OD% for each farm was calculated based on their four previous BTM samples, and also based on their four previous samples plus the 5th BTM sample that was collected in the calf study. The two mean calf OD% were calculated for each farm. Spearman Rho correlations were performed on the data in R (R Core Team, 2020) to test for an association between the mean BTM OD% for samples 1-4 and the apparent prevalence in calves tested, and BTM OD% 1-5 and the apparent prevalence in calves tested.

The second method categorised farms based on the test cut-off of 30% and 60% for the five BTM samples and calf samples, respectively. For the calf results, herds were categorised as negative, where there were no seropositive calves, and positive where there was at least one seropositive calf. Herds were categorised using one of the methods described in Chapter 5. Herds with all four or five negative BTM results were categorised as ‘negative’, those with all

positive BTM results were categorised as ‘positive’ and those with both positive and negative BTM results were ‘transitional’.

6.3 Results

6.3.1 Study participants

Thirty-six dairy herds participated in the present study, which was 44% of the total number of eligible herds that initially expressed interest in participating (n=81). Fourteen participating herds were consistently positive in the BTM study, nine were consistently negative, and 13 were transitional herds.

Twenty calf blood samples were received from all herds with the exception of one herd that sent samples from 16 calves, therefore the total number of calves that participated in the study was 716. A BTM sample was not received from two herds.

6.3.2 Prevalence estimations

Calf-level seroprevalence

Based on the ELISA test OD cut-off of 60%, 305 calves tested seropositive (43%) and 411 tested seronegative (57%). The individual calf OD values ranged from 0-311%, with a mean of 76% and a median of 8% (Figure 6-1).

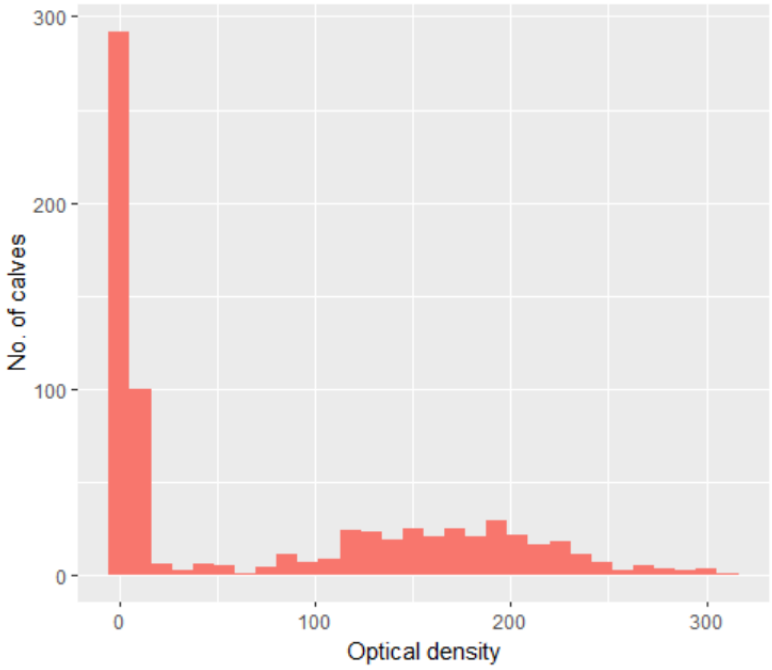


Figure 6-1: Distribution of ELISA optical density values of every calf in the study

Group-level seroprevalence

The seroprevalence of *M. bovis* by age group is shown in Table 6-1. The ages of calves were not provided for one herd, therefore the breakdown of seroprevalence by age group is based on 696 calves.

Table 6-1: Number of seropositive and seronegative calves by age group

Age	Positive	Negative	Total (seroprevalence)
4-8 months old	150	201	351 (42.7%)
10-14 months old	148	197	345 (42.9%)
Total	298	398	696

For both age categories, 43% of calves were seropositive and 53% seronegative. No difference in OD% was observed between the number of positive and negative 4-8 month-old calves and 10-14 month-olds ($p>0.05$) (Figure 6-2).

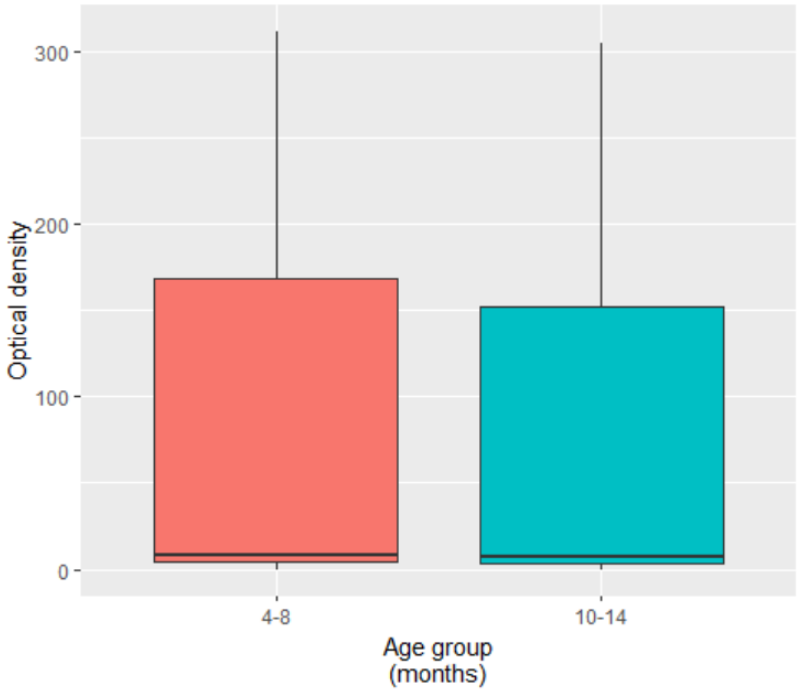


Figure 6-2: Distribution of ELISA optical density results by age group

Herd-level seroprevalence

The seroprevalence of calves within each herd varied, with some herds having all positive calves, all negative, and others a combination of both (Appendix 9, Figure 9-1). It is evident that in some herds there was a great variation in the OD values, whereas in others the OD values were similar among all calves tested.

The number of seropositive calves within the two age groups from each herd is shown in Appendix 9. There was an even split of 36 positive groups and 36 negative groups, with 19 of the 4-8 month-old groups negative and 16 positive groups. Sixteen 10-14 month-old groups were negative and 19 groups were positive. One herd did not state the age of their calves, one group was categorised as positive (7/10 positive) and one negative (0/10 positive).

Herds with at least one seropositive calf were categorised as positive (n=21) and those with no seropositive calves negative (n=15). Considering the sample size calculation, in the 21 positive herds, the seroprevalence was very likely to be ≥50%, and in the 15 negative herds, there was a 1% chance that the prevalence was ≥50%.

For further analysis, groups with one or more seropositive calves were categorised as positive and groups with no seropositive calves were categorised

as negative. The results of the two age groups in each herd were combined to categorise herds (Table 6-2). The majority of herds were either positive (n=15), i.e. at least one seropositive calf was detected in both age groups, or negative (n=15), i.e. no calves tested seropositive. In four herds, all 4-8 month-old calves were seronegative and at least one or more 10-14 month-old calves were seropositive. In only one herd, the younger age group were categorised as positive (n=9) and the older calves were negative. One farmer did not state which age group their calves were.

Table 6-2: Grouping of herds based on the age-group results: positive, negative, positive-negative, and negative-positive

Farm results	N (%)
Negative	15 (43)
Negative-positive*	4 (11)
Positive	15 (43)
Positive-negative*	1 (3)
Total	35

*The results of the 4-8mo calves-10-14mo calves

BTM

Twenty-seven BTM samples tested positive for the presence of *M. bovis* antibodies (79%) and only seven tested negative (21%). Two farmers did not submit a BTM sample.

All herds that tested negative in their 5th BTM sample (taken at the time of this study) also tested consistently negative for their first four samples in the BTM study (Table 6-3).

Table 6-3: Comparison of the BTM results for samples one to four in the BTM study and the fifth BTM sample in the calf study

BTM 5	BTM 1 - 4			
	Positive	**Negative**	**Transitional**	**Total**
Positive	13	1	13	27
Negative	0	7	0	7
Total	13	8	13	34*

*Two farms did not provide a fifth BTM sample in the calf study

The OD trends for every herd that submitted their fifth BTM sample is shown in the figures below. On observation of the previously grouped consistently positive herds, the OD% increased in their fifth BTM sample, with the exception of two herds that decreased, one closer to the test cut-off (Figure 6-3).

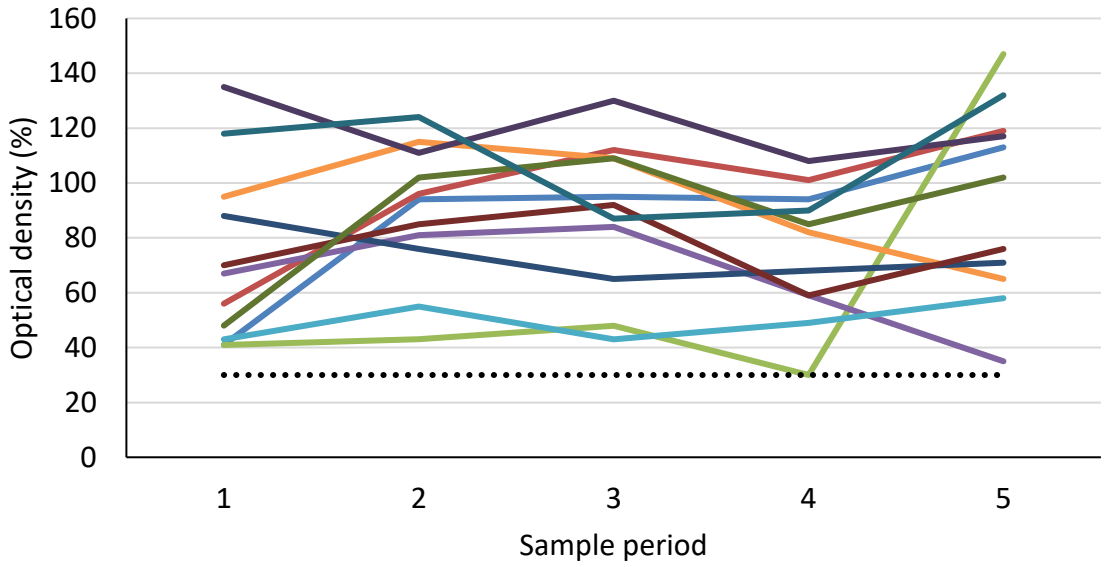


Figure 6-3: All five ELISA OD results for the farms classified as consistently positive in the BTM study. Test cut-off of 30% represented by dotted black line.

All herds that were categorised as consistently negative continued to decrease from sample four to five (Figure 6-4). The increase in OD% was very minor in one herd and it also remained negative. In the other two herds, the increase in OD% was much greater, with one nearing the test cut-off and one seroconverting to positive in sample 5.

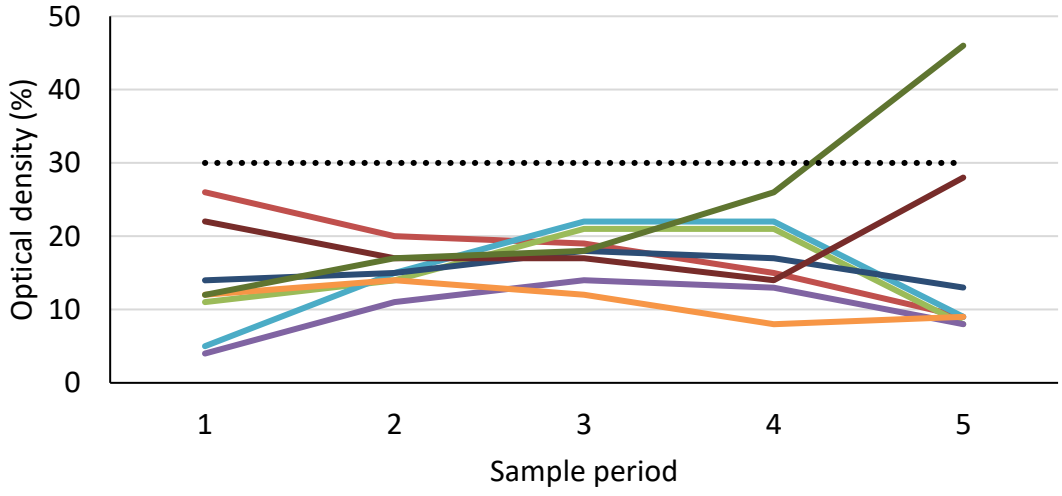


Figure 6-4: All five ELISA OD results for the farms classified as consistently negative in the BTM study. Test cut-off of 30% represented by dotted black line.

All herds that were previously classified as transitional tested positive in their fifth BTM sample irrespective of their previous four BTM samples (Figure 6-5). In two herds, their fifth BTM result was on or near the cut-off (30% and 33%).

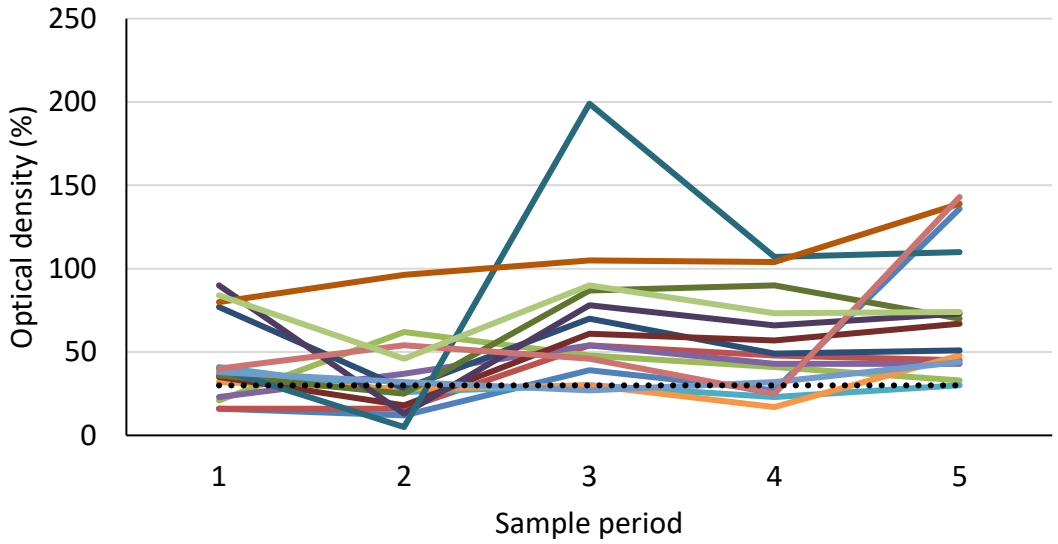


Figure 6-5: All five ELISA OD results for the farms classified as transitional in the BTM study. Test cut-off of 30% represented by dotted black line.

6.3.3 Association between antibody positive BTM samples and seropositivity in calves

Based on ELISA test cut-off

As shown in Table 6-4, herds were categorised based on the calf results as negative (both age groups negative), positive (both age groups positive), negative-positive (4-8 month-olds negative and 10-14 month-olds positive), or positive-negative (4-8 month-olds positive and 10-14 month-olds negative). The individual BTM results of the two herds that tested negative on all four BTM samples, and tested positive in one of the two age groups are shown in Appendix 9, Figure 9-2.

Table 6-4: Association between the BTM and calf herd-level ELISA results based on the test cut-off

Calves	BTM 1-5			Total
	Consistently positive	Consistently negative	Transitional	
Positive	9	0	6	15
Negative	3	6	6	15
Positive-Negative	0	1	0	1
Negative-Positive	1	1	2	4
Total	13	8	14	35

*One farmer did not provide the age of each calf and was therefore not included in this table.

Almost all herds that tested consistently negative in their BTM samples also tested negative in all calves (n=6), with the exception of two herds (one Positive-Negative and one Negative-Positive in the calves). Nine herds that tested consistently positive in their BTM had at least one positive calf per age group, in three herds all calves tested negative, and one herd tested negative in the 4-8 month olds and positive in the 10-14 month-olds. Six BTM transitional herds tested positive in both age groups of calves, six tested negative in all calves, and two tested negative in the 4-8 month olds and positive in the 10-14 month-olds.

Almost all herds that were categorised as positive in their calves also tested positive in their BTM results (n=15). One of the herds that tested positive in their calves only provided four BTM samples, and also the positive-negative herd only provided four samples.

Based on mean BTM OD% and mean calf OD%

Thirty-six dairy herds participated in the calf seroprevalence study, which was 27% of the total number of herds that completed the bulk milk prevalence study (n=133). Two farmers did not submit a fifth BTM sample at the same time as the calf blood samples, therefore 34 farms were included in testing for an association.

When comparing the first four BTM results to the calves, a positive association was observed between the mean BTM OD% and the mean calf OD% as shown in Figure 6-6 (p=0.014).

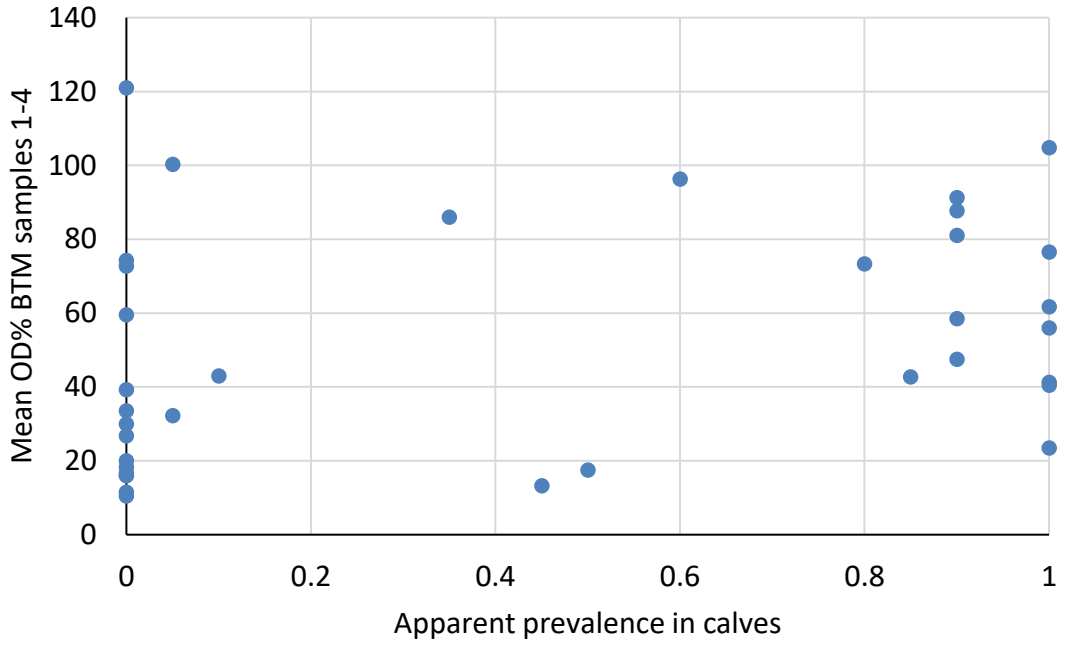


Figure 6-6: Association between the apparent prevalence of *M. bovis* antibodies in calves and the mean bulk tank milk sample (1-4)

Likewise, as shown in Figure 6-7, when all five BTM samples were compared to the calf results, there was a stronger positive correlation (p=0.003). As there was a stronger association when BTM sample 5 was included, a test for association was carried out between BTM 5 and the apparent prevalence in the calves. The association between BTM 5 and the apparent calf prevalences was very strong (p<0.001).

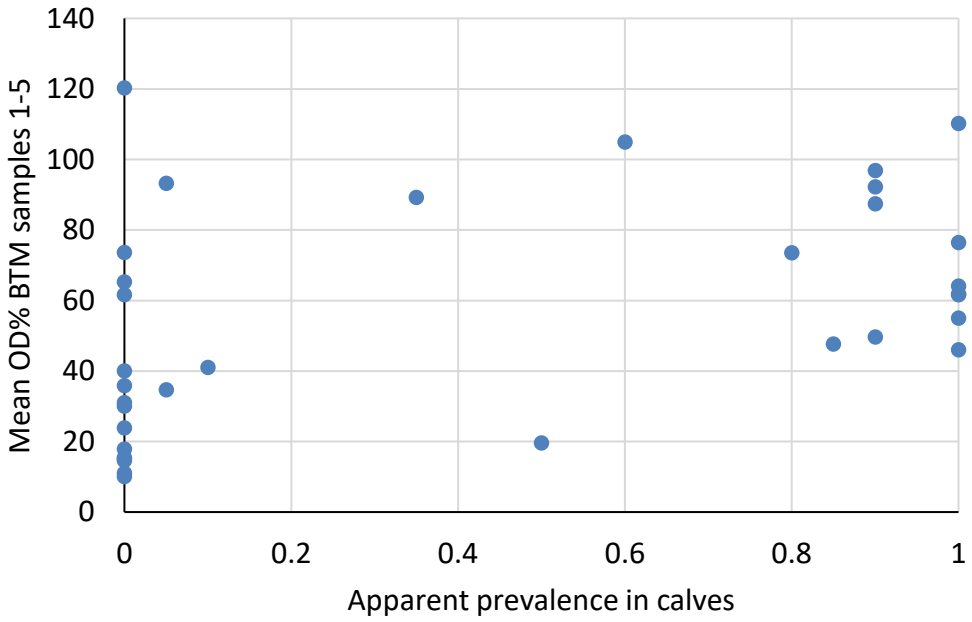


Figure 6-7: Association between the apparent prevalence of *M. bovis* antibodies in calves and the mean bulk tank milk sample (1-5)

6.4 Discussion

6.4.1 Calf seroprevalence estimates

This is the first investigation into the presence of *M. bovis* in Scottish dairy calves. The majority of herds followed the trend of 'all or nothing', i.e. all sampled calves were positive, or all were negative. Ten calves were selected for sampling from two age groups with the understanding that if one or more calves tested seropositive within a group then the likelihood that at least 50% had been exposed to *M. bovis* in that age group was very high.

Sampling from every animal in a herd is costly and not always practical, which is why sampling from a pre-defined number of animals is deemed sufficient. Herd screening is an efficient method that involves sampling from a proportion of a population to determine the frequency of disease, and to detect subclinical individuals (Torrence, 1997). Herd screening is carried out for various infectious pathogens including BVD virus. It was previously reported that for a high probability of detecting at least two positive BVD calves in a herd, only three animals need to be sampled (Hove, 1992). As part of the BVD eradication scheme in Scotland, five or ten calves are tested from each separate management group. Herds are categorised as 'negative' where no calves test seropositive,

'positive' if at least one animal has tested positive more than once by the antigen test (Scottish Government, 2019). BVD is not necessarily comparable to *M. bovis* as it is a virus that mainly causes gastrointestinal disease, whereas *M. bovis* is a bacterium causing respiratory disease. However, this study demonstrates that a similar approach could be applied to the surveillance of *M. bovis* in dairy herds, by testing a subset of the calves. Similarly, one study on bovine respiratory syncytial virus in Norwegian calves classified herds as positive if there was at least one antibody positive calf out of five sampled (Klem et al., 2013).

In the majority of herds categorised as positive, most or all calves within both age groups tested seropositive. In these particular herds that the majority of the calves in each age group (at least 50%) had been recently exposed to *M. bovis* and the disease may be widespread throughout the herd. Once *M. bovis* is introduced into a herd, preventing transmission within the rest of the herd can be challenging due to its ability to spread rapidly (Calcutt et al., 2018). Depending on the layout of the farm, most or all calf pens are likely housed in vicinity of each other enabling easy transmission of the pathogen as an aerosol between pens. Likewise, if divisions between pens allow for nose-to-nose contact, if pens share water troughs, or if feeding equipment is shared between calves, these factors will also facilitate between group transmission (Timsit et al., 2012).

There were a number of herds where the calves all tested seronegative throughout the two age groups. In these herds there was no evidence of recent exposure to *M. bovis* in either of the two age groups, however the presence of *M. bovis* could not be completely ruled out. Based on the sample size calculation, in these herds, the prevalence was likely to be less than 50%. The onset of clinical *M. bovis* disease in individual animals does not always result in a subsequent increase in antibodies (Maunsell et al., 2011; Petersen et al., 2018b), therefore antibody testing is not always recommended for detecting evidence of disease in individual animals. However, measuring antibodies at group-level has been shown to be much more effective at determining if calves have been recently exposed to *M. bovis* in beef cattle, and is likely the same in dairy calves (Martin et al., 1990). It is quite possible that *M. bovis* was not present in the study herds that tested negative.

A number of herds were an exception to the 'all or nothing' trend. In two herds that were categorised as positive in both age groups, all of the 4-8 month-old calves were positive and only one or two of the 10-14 month-olds calves tested seropositive. Another herd that was categorised as positive-negative had nine seropositive 4-8 month-olds but no positive 10-14 month-olds (Mbov_0063).

Farmers were asked to sample from calves as young as four months old. The target ages of youngstock to sample were 6 months and 12 months of age. To enable a large enough sample size, a range of two months was created around each age group, i.e. 4-8 months of age and 10-14 months of age. As discussed previously, maternal antibodies can exist in a calf up to and over six months of age (Chase et al., 2008). Though, the level of maternally derived antibodies in calves will depend on the nature of the infection within the adult herd, especially cows in late pregnancy. The IDvet ELISA cannot distinguish between maternally derived antibodies and antibodies from exposure (Andersson et al., 2019). Consequently, in the younger age group, it cannot be entirely assumed that seropositivity in this group is resulting from natural exposure to *M. bovis*. Thus, in those herds with few or no seropositive 10-14 month-olds and all seropositive 4-8 month-olds, it could be speculated that a proportion of the seropositivity is due to the presence of maternal antibodies in uninfected calves.

Additionally, one herd had all 10-14 month-olds that tested seropositive and all 4-8 month-olds tested seronegative (Mbov_0148). The persistence of *M. bovis*-antibodies is poorly understood, however few studies have suggested that they may be detected for some months post-infection (Byrne et al., 2000; Petersen et al., 2018a). In this particular herd, these results may suggest that there has been a historic infection. Alternatively, there could have been recent or current infection among the older age group and due to good hygiene practices and separate housing of the two age groups, there has been no opportunity for disease transmission between groups.

Three herds had only one or two calves that tested seropositive in total that were from the 10-14 month-old age group. These results were checked to ensure that they had been interpreted correctly. The sample size calculation stipulated that if no calves tested seropositive, then there was a 1% probability that the

population is seropositive at a prevalence of ≥50%. In these herds, the presence of *M. bovis* cannot be ruled out and it could be that the prevalence is very low.

The sensitivity of the IDvet ELISA test is very high at 95.7%, although when the test cut-off was optimised, the sensitivity was found to be 94.8% (Bokma et al., 2022). This is still a very high sensitivity, though there is a chance that in these herds, the one seropositive calf was in fact a false positive.

All 81 herds that completed three or four sampling points in the BTM study were asked if they wanted to participate in the present study, with the aim of sampling from 60 herds. Irrespective of their BTM results from the previous study, farmers that expressed their interest in the calf study may have believed that *M. bovis* was present in their youngstock which would make them more inclined to want to participate. Likewise, farmers that did not believe they had a problem with *M. bovis* in their calves might not have felt the need to participate. This could have led to an overestimation of the herd-level prevalence of *M. bovis* in youngstock.

Samples were sent out directly to selected farms and also to the vet practices of these farms depending on which was more convenient for the participants. The present study followed on from the longitudinal BTM study (Chapter 4), though there was some time in between the two studies where farmers would have finished the BTM sampling and would be waiting on their sampling kit arriving for the present study. This could have led to a proportion of farmers not wanting to continue participating as the momentum was lost. The challenges to maintain engagement in longitudinal studies are well established (Young et al., 2006).

Sampling kits were posted out to a number of farmers and vet practices, however blood and BTM samples were not returned. This may have resulted from kits being misplaced and forgotten about. Farmers were encouraged to collect samples during their next routine visit to avoid additional costs. In some instances, farmers may have not had their vet out for a routine visit during the sampling period, they may have forgotten about the study while their vet was visiting, or there may not have been enough time during the visit to collect samples for the study.

Farmers and their vet were asked to collect samples from the calves, and although instructions were provided, it was not known exactly how the sampling was conducted at each farm. For convenience, calves may have been sampled from only one or two adjacent pens rather than spreading out sampling across multiple pens. Most farmers did provide a rough schematic diagram illustrating the layout of their calf pens to enable us to see where the study calves were sampled from, and in most instances, calves were selected from a variety of different pens rather than all from the same pen. It cannot be strictly assumed that sampling was done as requested, however, based on the information provided by the participants it appears that most of the sampling was carried out well. Ideally, the researcher would have carried out the sampling, however this would have limited the number and location of farms included due to time and travel constraints.

Within study herds, the two groups were generated by dividing calves by to age ranges. *M. bovis* distribution within the youngstock in a herd can be quite batch-specific in nature, with one batch all testing seropositive while the other is seronegative, even within the same airspace (C. Mason, Personal Communication).

The two sampling groups can't be treated as homogenous as calves were sampled from different pens and therefore potentially exposed to different environments and stressors, i.e. if pens are housed across multiple sheds, different areas of a large shed, or if some pens are next to the lactating herd and the others are at the other end of the shed. Additionally, in many of the herds, calves of different ages were housed in the same pen, likely due to small numbers of calves and limited availability of space to divide them by age. Only a handful of farmers housed calves in groups with less than a one-month age gap (Chapter 3). Comparison of the results from the two age groups in each herd may be difficult as they may not be two truly distinct groups, which is why the results were also grouped at herd-level.

There are limited studies into the herd-level seroprevalence of *M. bovis* in dairy calves, with most youngstock studies focusing on beef feedlots. Sampling from 10 calves from each age group could be a quick and efficient approach to capture the presence of *M. bovis* antibodies in youngstock. Though this has not

yet been validated, i.e. by testing all calves in the herd and then testing 10 to determine if sampling from 10 calves is suffice. This would be an appropriate next step in terms of *M. bovis* research in Scottish dairy herds and would improve veterinary diagnostics. The difference in results between the two age groups suggest that with good biosecurity, the spread of *M. bovis* can be minimised or prevented between groups of calves.

6.4.2 BTM results

The majority of BTM samples from the study herds tested positive for the presence of antibodies to *M. bovis* suggesting that the lactating cows in these herds were recently exposed to the disease. BTM sampling is a quick and easy disease detection tool that provides a snapshot that day. Farmers can sample from the bulk tank and a single sample can be tested for a variety of disease making this a cost-effective method of testing.

A number of factors can influence the BTM test results, the main factor being that a BTM sample contains milk only from cows that contributed to that particular sample. Cows will enter and leave the lactating herd for various reasons, and this may alter the BTM test result, as shown in Chapter 5, Figure 5-20. New first calvers could be introduced the day after sampling who were positive and could have changed a BTM result from negative to positive. Cows will not be contributing to the bulk tank if they are suffering from clinical mastitis or receiving antibiotic treatment, and dry cows will also not be contributing to the tank but may have been recently exposed to *M. bovis*. This is why repeated BTM sampling is better as it the changes in cows that enter and exit the lactating group will be captured.

When comparing the results of BTM 5 to the previous four BTM samples, all herds that tested negative in their most recent BTM sample also tested negative in their previous samples. In those herds it could be said that there has been no exposure to *M. bovis* for well over one year. It also demonstrates that with good biosecurity measures, farmers can avoid introducing *M. bovis* into their herd.

6.4.3 Association between BTM and calf seroprevalence

Associations were seen between apparent calf prevalence and both BTM samples 1-4 and samples 1-5. Although BTM sample 5 was sampled at the same time in which the calves were sampled, the previous four BTM samples were sampled closer to the time when calves were likely exposed to *M. bovis* on a more regular basis. The study calves were aged between 4-14 months of age, which means that the previous four BTM samples may in fact be better for comparison as the calves were more likely to have direct contact with the milking herd during those BTM sampling periods. However, when testing for an association between the calf seroprevalence and BTM 5 alone, there was an even stronger association. This possibly suggests that the within farm biosecurity between adults and youngstock allows for transmission between the two groups of cattle. Thus, it is important to consider both groups of cattle when investigating the spread and maintenance of *M. bovis* within a herd.

As described in Chapter 1, *M. bovis* transmission between cows and calves is possible via numerous routes such as direct contact, during calving, via milk and colostrum, as an aerosol, and via fomites such as farmers' clothing. A recent study investigated the within-herd transmission of *M. bovis* between cows, youngstock and calves in Dutch dairy herds (Biesheuvel et al., 2024b). The authors reported that cow to cow, cow to youngstock and cow to calf transmission were the most significant contributors to the spread of the disease within herds, followed by calf to calf and calf to youngstock transmission. These findings support the idea that there is a strong link between the exposure of *M. bovis* in calves and the infectious status of the lactating herd. Further research is required to better understand the timing, direction and routes of spread, and ultimately the effectiveness of different management practices and biosecurity measures on the infection dynamics.

Herds that tested consistently positive in their BTM samples and tested seropositive in their calves have likely had recent or ongoing infections with *M. bovis* throughout the entire herd. *M. bovis* is a highly transmissible pathogen and once the disease is introduced into a herd it spreads readily between individuals, and between groups of animals.

A previous study in Danish herds reported no association between the BTM OD% and the seroprevalence of *M. bovis* antibodies in youngstock (Petersen et al., 2016). The reason that an association was not observed between the youngstock and BTM in the Danish study could be due to the fact that *M. bovis* appears to be more prevalent in Scotland compared to Denmark (Kusiluka, Ojeniyi and Friis, 2000). Herds in Scotland will have been infected for a longer period of time, and thus the disease will have filtered down from cows to calves. There could also be better biocontainment within Danish dairy herds that are minimising the potential for *M. bovis* transmission from cows to calves.

Herds that test consistently negative in BTM samples collected over a 12-month period have likely not experienced an outbreak of *M. bovis* in the lactating herd. Where subsequent sampling of the youngstock showed no evidence of exposure, it could be assumed that these herds are free from *M. bovis* disease. BTM sampling only provides a snapshot of the cows that contributed to the tank at the time of sampling, therefore, it is much more reliable to collect repeated samples and observe the overall trend.

Only two herds had seropositive calves and negative BTM results. In one of the herds, the 4-8 month-olds were all seronegative and all of the 10-14 month-olds were seropositive. The BTM trend of this farm is shown in Appendix 9, Figure 9-2. Due to the fact that all individuals in the older age group tested seropositive this cannot be attributed to false positives. Since *M. bovis*-antibodies were not detected in the BTM, this could suggest that the farmer practiced good hygiene and minimised the risk of disease spread between groups, and this may have been the remnants of a historical infection. Alternatively, *M. bovis* may have been recently introduced into this herd via bought-in asymptomatic youngstock and the infection has not yet spread between groups. The latter is plausible as the BTM OD% for each of the five samples from this herd decreased between samples 1-4 and then increased in sample 5. Although sample 5 tested negative, the OD was marginally lower than the test cut-off. There may have been some antibody positive cows in the milking herd, but not enough to push the OD% above the cut-off. As discussed previously, the duration of antibody detection post-infection is poorly understood and may be detectable for a short period of time or for many months (Hazelton et al., 2018b; Hirth et al., 1966). Until the persistency of antibodies following *M. bovis* infections is better understood, it is

difficult to draw conclusions as to whether a herd was previously or is currently experiencing active infections based on antibody testing. Though, it could be hypothesised that herds with high antibody seroprevalence have active infection present.

In the other herd with a history of negative BTM, almost all of the 4-8 month-olds were seropositive and the 10-14 month-olds were all seronegative. This farmer also only provided four BTM samples (trend shown in Appendix 9, Figure 9-2) and did not submit a sample for testing with the calf samples. In this herd it could be surmised that *M. bovis* has been recently introduced into the herd which is why all of the previous BTM samples tested negative. The younger age group will have been more recently exposed to any diseases from the lactating herd via milk and colostrum which would explain why only the younger group tested seropositive. It would have been beneficial to have received a more recent BTM sample to test as this may have shown that the lactating herd have also been recently exposed to *M. bovis* and support this theory.

Six herds tested positive in their BTM samples and negative in all the calves. This could suggest that these herds have good hygiene practices that prevented the spread of *M. bovis* between the cows and youngstock. Further investigations would be required to determine if there is any evidence of exposure in the youngstock in these herds. As there are conflicting findings in previous studies testing for associations between BTM and youngstock prevalence, further research is required to fully understand this association and identify the most important routes of transmission between the two groups of cattle. Feeding of milk and colostrum from infected cows was not associated with the prevalence in both the BTM and calf studies, and previously it was believed to be a risk factor for *M. bovis*. If *M. bovis* is not spreading as readily in cows' milk and colostrum, it may be spread via fomites and farm workers. Furthermore, studies are required to identify potential risk factors associated with seropositivity in youngstock, as this information could be used to advise farmers on approaches to minimise or prevent the risk of introducing and spreading *M. bovis* in their herd.

6.4.4 Limitations of the study

Three main limitations were apparent in the present study, related to the selection of participants, the funding available, and maintaining momentum.

One of the limitations of this study was that the herds were pre-selected and not a random sample of the total dairy population in Scotland. Therefore, it was likely not a representative sample of Scottish dairy herds. The aim was to recruit an even split of 20 herds from each of the three categories identified in Chapter 5: consistently positive, consistently negative, and transitional. Although the funding allowed for sampling from 60 herds in total, only 36 herds participated in the study, 14 consistently positive, nine consistently negative and 13 transitional. At the time of promoting the calf study to farmers, they were ready to send their fourth and final BTM sample for the longitudinal BTM study (described in Chapters 4 and 5). In total, 81 farmers were initially interested in participating, most of which were from the 'consistently positive' classification. However, the focus was put onto recruiting as many of the negative and transitional herds to ensure that these groups were represented. Once farmers from the selected consistently positive herds were contacted, the momentum may have been lost, which was why many of the farmers did not decide to participate in the calf study. The number of herds in the study was 4% of the total dairy herd population in Scotland (843, SDHA, 2021). The aim of this study was not to estimate calf seroprevalence in Scottish herds, but to study the relationship between BTM and calf prevalence on farms with varying known *M. bovis* status.

In terms of the number of calves sampled per group/farm, the sample size informed how many calves should be sampled in each herd to achieve 99% confidence that at least one calf would test positive if the prevalence in the population was >50%, based on an estimated population size of 100. Only one of the study herds had just over 100 animals in the 10-14 month-old age group, and all the rest of the herds had fewer than 100 animals in each of the two age groups. Therefore, the strategy of sampling 10 animals per group provided sufficient power to be 99% confident that if *M. bovis* was present in the group at a minimum prevalence of 50% then at least one sampled calf would be positive.

The results may not be representative of the total Scottish dairy herd population, thus further research is required to estimate the prevalence of *M. bovis* within dairy calves in Scotland and to explore the association between the BTM and calf prevalence.

6.4.5 Conclusions

This was the first study investigating *M. bovis* in youngstock in Scotland. The results showed that there was evidence of exposure to *M. bovis* in most study herds. There can be differences in the results when sampling from 4-8 month-olds and 10-14 month-olds, i.e. one group positive and the other negative, which is why it is important to get a good representation across a range of age groups, particularly in larger herds. As mentioned previously, the validity of sampling from 10 calves within an age group has not yet been tested, therefore, to utilise this approach to screening, sampling from 10 calves needs to be validated.

The study as demonstrated that there was evidence of exposure to *M. bovis* in most herds and was associated with the prevalence in the BTM. Further studies should investigate the association further and identify routes of transmission between the lactating herd and youngstock.

Chapter 7 General Discussion

This thesis was developed to improve our understanding of *M. bovis* in Scottish dairy herds by conducting two studies: a longitudinal BTM prevalence study and a cross-sectional calf seroprevalence study.

7.1 Overall picture of *M. bovis* in Scotland

The studies that form this thesis were an assessment of the prevalence of *M. bovis* in Scotland, as there had been no research carried out previously. The point estimates for true BTM prevalence of *M. bovis* antibodies across the four sampling points ranged from 74-87%, and 86% of the study herds tested positive in at least one of their four BTM samples (Chapter 4). Furthermore, based on the ELISA test cut-off, over 60% of herds tested consistently positive for the duration of the study. These findings suggest that *M. bovis* is likely to be endemic in Scotland.

Balancing the internal and external validity can be difficult (Pannucci and Wilkins, 2010), however can be achieved by minimising the potential for bias and using a representative sample of the total population. Many factors were considered that may have impacted the internal validity of the BTM study. As discussed in Chapter 3, in both the BTM and calf studies the potential for recall bias cannot be ruled out. In questionnaires, participants are required to recall information or provide information from records, for example herd vaccination records (Dörnyei and Taguchi, 2009). In both questionnaires issued to farmers, the majority of questions could easily be answered by farmers as they related to the farm layout and management practices. Only a couple of questions asked farmers to provide numbers of animals in different age groups, including the numbers purchased over the previous 12 months. Some farmers may have estimated these figures. Recall bias can result in the underestimation or overestimation of an association between an exposure and an outcome in the study population due to misclassification of the exposure (Jager et al., 2020). An example of this could be that if multiple farmers believed they had purchased cows in the previous 12 months when they had not. This could have resulted in an overestimation of the association found between the BTM prevalence of *M. bovis* and buying in cows in the BTM study.

Similarly, reported behaviours may differ to the actual behaviours/actions of participants. Where invasive or sensitive questions are asked, participants may wish to respond with an answer that they deem is the best or most appropriate, rather than what is true to what they believe or practice themselves. In both questionnaires, no questions were on particularly sensitive topics that would have driven farmers to provide a false answer. Although there were no sensitive questions, farmers may have answered based on what they intended to do rather than what they actually do in practice. For example, in the BTM study, Q13 asked farmers how often they clean and disinfect calf feeding equipment. Clean feeding buckets, tubes and teats are essential to prevent the risk of infection and disease spread, and this is a well-known fact to anyone who rears livestock. Farmers may intend to clean feed buckets every day knowing that this is best practice, but in reality, they are cleaned a couple of times per week, and thus they answer: 'cleaned every day'. Again, this could have had an impact on the associations observed between the exposure and outcomes.

Another aspect relating to the internal validity of the study is trying to capture the complexity of management practices in the herds. An example of this was in Q13 of the BTM study where farmers were asked how they clean and disinfect calf feeding equipment. A list of pre-defined choices was provided though it was evident upon observation of the returned questionnaires that this question was too complex. Cleaning and disinfecting are two separate tasks and combining them in one question and caused confusion among the farmers. This was an extreme example that resulted in the question being unusable for data analysis, however this may have occurred in other questions. When pre-defined choices are provided in a questionnaire, it could result in participants selecting the response that is closest to their situation. The results to both questionnaires highlighted that although there were common practices among many of the dairy farms, there was also a variety of practices and structures captured among the 181 study herds. It is possible that in some of the questions, the intricacies of individual herds were not captured. Thus, there may have been an over- or under-estimation of an association between an exposure and outcome as participants were not able to give the correct answer.

External validity was assessed by studying the representativeness of participants with the target population. Whilst the geographical distribution and average

herd size appeared representative, it was not possible to assess other farm attributes such as average yield due to a lack of comparative population data. In terms of whether the results could be extended to other systems (e.g. beef sucklers), it was hypothesised that risk factors may be similar due to the biological processes. Although, they may differ in behaviour and management such as purchasing cattle and cattle movements (Biddle et al., 2003; Bishop et al., 2010). If a herd has experienced a previous or recent outbreak of *M. bovis*, the risk of *M. bovis* being continually detected throughout the herd increases.

As illustrated in Figure 5-10 (Chapter 5), cows will be entering and leaving the lactating herd throughout the year. A proportion of herds had transitional BTM trends, either seroconverting, sero-reverting, or fluctuating back and forth between positive and negative, demonstrating that *M. bovis* antibodies can be dynamic in nature. Within the space of three months, the BTM OD% can change drastically, highlighting the importance of regular testing. Despite the changes in cows contributing to the BTM throughout the study period, the BTM antibody OD% remained fairly stable in almost half of the study herds. Within those stable herds, many remained positive for the duration of the study. If a herd with mastitis due to *M. bovis* is continuously testing antibody positive, then further testing of individual animals could be done to identify cattle that are the source of infection. In those herds it is evident that the disease is being maintained within the herd, and multiple groups of animals (shown in Figure 5-10) are introducing *M. bovis* antibodies into the BTM when they enter the lactating group.

There was evidence of exposure in the youngstock in 58% of the farms that participated in the calf study. As discussed in Chapter 6, although there were several herds in which all calves tested seronegative, this does not mean that there was no evidence of exposure to *M. bovis* within those herds, rather that the likelihood of the seroprevalence being 50% or more was very low. The BTM study also demonstrated that the BTM antibody result is a good proxy for the presence of *M. bovis* antibodies in youngstock. There was a strong association between the BTM results and the mean calf results, both when comparing the results based on the ELISA test cut-offs and the OD results. Though this was conducted on a small sample size of only 36 farms, there was still a clear trend, with higher BTM OD% coinciding with higher mean OD% in the youngstock. This

could suggest that there is transmission of *M. bovis* within the herd, potentially via milk and colostrum, fomites, or farm staff.

In Chapter 4, potential risk factors associated with a positive BTM sample were identified. These were the history of *M. bovis* in the herd, rearing dairy bull and beef calves, and cow housing. If *M. bovis* is currently, or has recently been, present in a herd, then antibodies would be detectable in the BTM for a period of time afterwards. The other two risk factors both relate to the density of animals on the farm. Indoor housing tends to be more densely populated compared to grazed cows, thus increasing the risk of disease spread. Similarly, if herds are rearing dairy bull and beef calves, this increases the number of susceptible animals in the herd, again increasing the potential for *M. bovis* transmission. Further research is required to understand these risk factors more and to identify others.

Observational studies generate hypotheses instead of testing hypotheses (DeWees et al., 2019) and are an appropriate first study to conduct when there have been no previous investigations on a specific disease within a population. In terms of next steps, future research should focus on within-herd prevalence in Scottish dairy herds. It would be beneficial to know the relationship between how many antibody-positive cows, or rather what proportion are positive, and the BTM seropositivity. This would improve the value of testing BTM as farmers and vets would then have an idea of how widespread *M. bovis* may be within the herd if they had a BTM OD% of, for example, 45% or 145%. Though, it could be argued that this information is now not as important due to the availability of vaccines. Further studies on dairy calves should be conducted with larger sample sizes to provide better estimates of *M. bovis* prevalence and identify potential risk factors. The association between the BTM and youngstock antibody results observed in Chapter 6 should be investigated to further understand how strong the association is and the factors that link the BTM and youngstock, such as feeding of cows' milk, housing setup, hygiene practices, and farm staff.

Until such time that *M. bovis* is incorporated into a national health scheme or a control programme is established, the onus will be on farmers and vets to monitor the presence of *M. bovis* in dairy herds. The overall findings suggest that *M. bovis* is likely endemic in Scotland.

7.2 What's next for Scotland?

This section explores the possible next steps for managing and monitoring *M. bovis* in Scottish dairy herds. As it stands there are different avenues the country could go down, though almost all options require further understanding of *M. bovis* prior to considering them.

Business as usual

The results of the BTM study have shown that *M. bovis* appears to be highly prevalent in the Scottish dairy industry. Though, as discussed in Chapter 4, it is not known how much of an impact the disease has within herds in Scotland. There is only one study to date that has assessed the impacts of *M. bovis* on productivity and profitability (Timonen et al., 2017). The authors reported that the within-herd prevalence of *M. bovis* (based on PCR testing of individual cow milk samples) was 17.2% and *M. bovis*-positive cows produced 3.0kg less milk per day than negative cows. If this estimate of the reduction in daily milk yield was applied to the current situation in Scotland this could work out at a loss of around £10,000 per year if 20% of the herd were infected (see Appendix 10-1). This is based on a 100-cow herd, an average daily milk yield of 30l per cow and 44 pence per litre (AHDB Dairy, 2024).

This is a significant financial loss to infected dairy farms and does not take into consideration the cost of treatment, vet costs and other potential production costs associated with *M. bovis* presence. Though, it should be noted that this is a simple costs calculation that does not factor in the reduced feed intake due to lower milk production. This estimate is based on a within-herd prevalence of 20%, however the within-herd prevalence of *M. bovis* mastitis may be much lower in Scotland. The reduction in daily milk yield due to *M. bovis* may also be lower in Scotland as *M. bovis*-mastitis does not appear to be a major clinical disease, therefore the estimated financial loss is likely the absolute maximum that could occur in Scottish dairy herds due to *M. bovis* mastitis.

To the author's knowledge, there have been no other studies to date that have quantified or assessed the impact of *M. bovis* on specific production parameters nor the cost of having the disease in dairy herds.

As *M. bovis* causes more pneumonia rather than mastitis in the UK, it would be beneficial to estimate the potential impact of the disease in calves. The estimated cost of pneumonia (from all causes) in youngstock was calculated in a herd with 100 dairy bull beef calves (see Appendix 10-2). The annual profit from a herd with pneumonia in youngstock was estimated as £4,811, compared to £15,700 with no pneumonia.

There is currently no within-herd prevalence estimate of *M. bovis* specifically in Scottish or UK dairy herds. This knowledge would provide evidence to support the development of any future control strategies or monitoring activities as currently there are none for *M. bovis* in Scotland, or in the UK. Passive surveillance of *M. bovis* occurs via samples tested through the Animal and Plant Health Agency's (APHA) Veterinary Investigation Centres and other partners throughout the UK. The data generated from this passive surveillance only represents samples that have been sent to these post-mortem centres for diagnostic testing, therefore, any trends or changes seen over the last decade may not truly reflect the national situation. Since 2015, the proportion of *M. bovis* submissions increased from <10% to >20% in 2021 and 2022. This increase coincides with an overall increase in our awareness of *M. bovis* as well as advancements in diagnostic tests for the disease.

Prior to the introduction of commercial vaccines in 2024, *M. bovis* was controlled by managing risk factors and treating clinical disease where needed. Prevention and monitoring of *M. bovis* often requires a comprehensive control strategy involving testing, vaccinating and management changes. A farmer's experience with *M. bovis* will ultimately influence their decision to take preventive action against the disease. Additionally, vets may advise their clients to consider testing for *M. bovis* if they have other clients impacted by the disease.

To minimise the risk of *M. bovis* introduction to a herd, farmers should continue to practice good biosecurity including operating a closed herd system or testing and quarantining any animals that are purchased. It has been documented that *M. bovis* can be introduced into herds via semen from infected bulls (Haapala et al., 2018) and a previous study demonstrated that herds with breeding bulls were more likely to have a positive BTM sample than herds that did not have a breeding bull (Gille et al., 2018). This association was not observed in the present study, though as explained in Chapter 4, this may have been due to

M. bovis being more endemic in Scotland compared to other countries. Although many farms keep breeding bulls, these bulls are typically used as 'sweepers' to serve cows that are not pregnant after one or two rounds of AI. Therefore, even with breeding bull testing, there is still a small risk of *M. bovis* introduction via AI. Currently, semen at AI banks is not screened for *M. bovis* though semen extenders do contain antibiotics which should minimise the risk of *M. bovis* survival and transmission via the semen (Haapala et al., 2018). Semen banks could test for *M. bovis* as this would enable farmers to address the risk of both natural breeding and the use of AI as potential routes of *M. bovis* introduction, however it is not likely that this will be implemented anytime soon. For now, farmers can only quarantine and test any purchased breeding bulls for *M. bovis*.

Once *M. bovis* enters a herd, farmers can adopt good biocontainment measures to prevent disease spread throughout the herd. The routes of transmission in a herd are shown in Figure 7-1. There are various areas that can be targeted by farmers to prevent the spread such as avoiding feeding waste milk to calves, pasteurising cows' milk and colostrum that is fed to calves and adopting good colostrum management. Though one recent study reported that only 1.9% of colostrum samples tested positive for *M. bovis* from herds recently infected with the disease (Gille et al., 2020), which suggests that there may be a need to reconsider if milk and colostrum are significant routes of transmission.

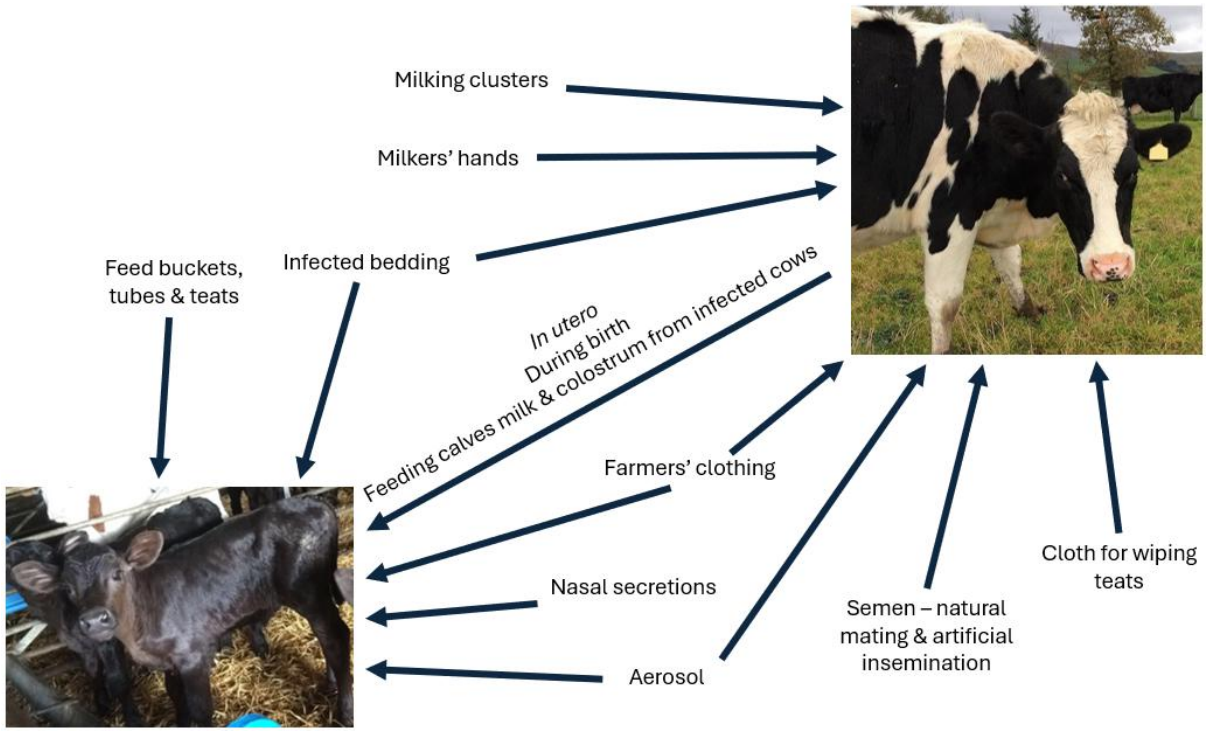


Figure 7-1: Within-herd transmission routes of *M. bovis*.

M. bovis is also known to survive in the environment for months post-infection (Justice-Allen et al., 2010). Therefore, farmers should continue with good hygiene practices during milking such as regularly washing their hands, wearing gloves and using separate cloths to wipe teats of each cow before milking. Feeding equipment and bedding should be cleaned and disinfected regularly.

Autogenous vaccines are also an option to farmers, and prior to the production of the two commercially available vaccines Myco-B™ (American Animal Health, Grand Prairie, Texas, USA) and Protivity® (Zoetis, USA), they were the only option available to protect herds against *M. bovis*. Autogenous vaccines are made using the specific strain within the herd, which can make them more effective at preventing the disease, particularly in closed herds. As the main clinical disease caused by *M. bovis* is respiratory disease in youngstock in the UK, autogenous vaccines can be less favourable in calf rearing units where cattle are housed from multiple farms and were previously exposed to potentially different *M. bovis* strains. Some farmers may continue to use autogenous vaccines if it has been successful at reducing or eliminating *M. bovis*, however it is likely that most farmers who decide to vaccinate against the disease will use one of the two commercially available vaccines.

Research has so far shown that there is a significant diversity of strains of *M. bovis* in Scotland (unpublished data). Until such time that a new strain of *M. bovis* emerges in Scotland which has a higher mortality rate, causes more serious clinical disease, and has a significant impact on the dairy industry (production, welfare and financial), there is no real need to establish a national control or monitoring strategy, nor is nationwide eradication required. Furthermore, the prevalence in Scotland, and likely the UK, is too high to merit national control and eradication. Though, this does not mean that farmers should not implement changes to their herd management to prevent *M. bovis* entering their herd or to control and eliminate it.

Commercially available vaccines

There are currently two new commercial vaccines available in the UK for protection against *M. bovis*: Myco-B™ and Protivity®. Both vaccines are described by their manufacturers as being effective at reducing the rates of *M. bovis*-associated respiratory disease and arthritis. Myco-B™ is a single shot vaccine that can be administered to dry cows and calves from 60 days of age and

above. This vaccine does not require a booster which reduces the cost and labour required to administer a second dose. Protivity® is a modified-live vaccine that requires two shots 21 days apart and is given to calves only. The advantage of Protivity® is that it can be administered to calves as young as one week of age.

Prior to November 2024, these commercial vaccines were only available in the UK on a special import license. This change in the availability of these vaccines is very positive for the UK dairy and beef industries and will hopefully be utilised by farmers and vets. The vaccines are particularly important for protecting naïve animals.

In the calf study (Chapter 6), the range in calf serology results between and within herds was notable. In most herds the sampled calves were either all positive or negative. Where the entire study cohort tested positive, this indicated that there was exposure and circulation of *M. bovis* throughout the herd. In the entirely negative herds, there was no evidence of exposure to the disease, suggesting a naïve status. In other herds, the serology results differed between the two age groups sampled, and in some instances, there were differences within the age groups, as shown in Figure A9-1, Appendix 9.

The variation in serology status of calves within herds in the calf study highlights the importance of using vaccines to minimise the spread of *M. bovis*. The range of ages that *M. bovis* commercial vaccines can be administered to calves (from one week of age with Protivity® and 60 days of age with Myco-B™) gives farmers the ability to tailor their vaccination approach depending on *M. bovis* disease dynamics within their herd and to target different age groups if necessary.

Targeted screening and risk-based herd accreditation

Introduction to targeted approaches

Another option for *M. bovis* monitoring and control in Scotland is through targeted screening and herd risk rating. Implementing a national monitoring strategy to all herds would not be practical, nor feasible. Instead, a targeted approach would identify high risk herds, enabling a better use of time and resources and prioritisation of areas that need monitoring. The identification of high-risk herds for targeted screening can be achieved by using known risk factors and surveillance data. As previously discussed, there are various risk

factors associated with the presence of *M. bovis* such as herd size, stress and calving setup. Also, three further risk factors were identified in this thesis: herd history, rearing of dairy bull beef calves and housing of the lactating herd. As a first step, herds that are at increased risk due to their structure or management practices could be sampled.

In Scotland, surveillance data on *M. bovis* is limited as there is no active surveillance, however, passive surveillance occurs through veterinary disease surveillance centres throughout the country. This data could be used to observe patters of disease and determine regional areas that are most at risk.

This information could be used to evaluate the infection status of individual animals or herds and thus assign a risk rating to the herds. Once assigned a status, farmers and their vets can tailor the necessary interventions to their herd.

Targeted screening and risk-based accreditation may be fundamental to monitor the presence of *M. bovis* in Scottish dairy herds and to protect naïve animals and herds from the disease.

Screening and sampling strategies

The BTM study (Chapter 4) demonstrated that testing quarterly BTM samples for the presence of *M. bovis* antibodies provides a good representation of the herd-level disease dynamics over the year. Collecting BTM samples is quick and can be done by the farmer, thus reducing the cost of a vet call-out fee, and one sample can be tested for various pathogens, again cutting costs. As discussed in Chapter 4, there are some limitations to testing BTM samples by PCR for the presence of *M. bovis* DNA. One of the main limitations is that *M. bovis* is shed intermittently, paired with the fact that DNA from individual cows will be diluted in BTM, making it challenging to detect active infection. This is why using both PCR and ELISA testing in the BTM will provide the most accurate representation of the herd disease status.

As discussed in previous chapters, it is not known what proportion of positive cows need to contribute to the BTM in order to obtain a positive sample, nor the implications of different OD values, for instance whether an OD of 40% or 180% indicates a widespread presence of antibodies within the herd. Therefore, further research is required to establish the significance of varying positive OD values.

As presented in Chapter 4, 20 herds (15%) tested consistently antibody-negative in all four BTM samples. Six of those tested negative in their 5th BTM sample and all 20 calf samples taken in the calf study (Chapter 6). In these herds, there is no evidence to suggest that *M. bovis* is present. Negative herds appear to be less common, therefore these herds should be protected with good biosecurity practices to prevent the introduction of *M. bovis*. Furthermore, if negative herds sell their dairy bull and beef calves to rearing facilities, these calves should be vaccinated to protect them from *M. bovis* exposure.

As well as testing the BTM, calf screening provides insight into the presence of *M. bovis* in the herd. In the calf study (Chapter 6), up to 20 young animals were sampled from each herd. The antibody levels among calves in the same herd were variable, with some study herds testing entirely positive, some all negative, and in others there were both positive and negative calves. This highlights the importance of a good sampling strategy to ensure the sampled population is representative of the whole herd. As discussed in Chapter 6, the structures and management practices differed among farms, which could influence the screening approach. For instance, if there are fewer calves in the herd at one time (i.e. in smaller or year-round calving herds), it may be possible for farmers to sample all calves. Whereas in larger herds only a subset may be sampled.

Herd accreditation

Currently, *M. bovis* is not included in any voluntary health scheme, nor is there any form of herd accreditation. If this was initiated for *M. bovis* in Scotland, it would help manage the prevalence of the disease and minimise the risk of spread between herds.

As discussed in Chapter 5, Scottish farmers can enrol onto voluntary herd health schemes to improve the health, welfare and productivity of their herd by screening for the most significant single-agent cattle pathogens in the UK. Enrolment in these programmes requires initial testing to ascertain the herd's disease status, followed by biosecurity measures and regular testing of a combination of individual animals >1-2 years old, youngstock, BTM, and non-homebred animals. Herds are assigned an accreditation level, for example free from disease, monitored and eradication, or low, medium and high risk.

The design of a potential *M. bovis* health scheme could be based off of the already existing schemes and follow a similar structure or accreditation approach. Herds could achieve different levels of accreditation from regular screening as described above. Farmers may also be required to operate specific management practices to optimise biosecurity and biocontainment such as having a closed herd or testing purchased animals, vaccinations, and ensuring good hygiene standards during milking and calf rearing.

Prior to commencing active surveillance or establishing a herd health scheme, there would need to be a method of assigning the disease status to herds. Based on the BTM and youngstock results from the calf study (Chapter 6), the 36 herds could be assigned to one of the following statuses:

1. Highly likely that *M. bovis* is present throughout the herd
2. *M. bovis* is likely present in some of the herd
3. Little evidence to suggest that *M. bovis* is present in the herd

For category 1, this could contain herds that tested antibody positive in both the BTM and the youngstock. Herds that tested antibody positive in their BTM samples but negative in the youngstock, or vice versa, could be assigned to category 2. Category 3 could contain herds that did not test antibody positive in their BTM sample or in the youngstock. If this was applied to the 36 herds in the calf study, it would be highly likely that *M. bovis* was present in 18 herds, likely present in nine herds, and little evidence to suggest *M. bovis* was present in six herds. This accreditation approach could be applied to the dairy herd population in Scotland and would enable farmers to tailor control strategies to their herd depending on their status. In herds assigned a low-risk rating, farmers may implement better biosecurity measures and vaccination to prevent the introduction of the disease. There is possible scope for farmers to sell and purchase negative, vaccinated, animals. In herds where there is a medium or high risk of *M. bovis*, farmers may opt to cull infected animals, alongside the measures for low-risk herds.

To optimise the uptake of an accreditation scheme for *M. bovis*, this would require engagement with stakeholders, farmers and vets (e.g. workshops, newsletters and communication with industry groups) to raise awareness, and an offer of financial support to those participating. Facilitating any means possible to utilise existing resources would reduce the input required by farmers and

increase uptake. For example, routine testing of BTM already occurs via milk recording laboratories, therefore, *M. bovis* testing could be incorporated this. Also, the process of submitting samples to veterinary diagnostic labs is well established, therefore it would be easy to incorporate *M. bovis* testing to existing submissions. Furthermore, *M. bovis* could be included in current health schemes, though an estimation of the cost of regular testing and changes to management practices must be known prior to the inclusion of the disease.

Screening for *M. bovis* cannot follow a 'one-size-fits-all' approach. Control and prevention strategies will need to be tailored on a case-by-case basis, targeting groups that are affected, guided by both the farmer and their vet.

Eradication from Scotland

Eradication from Scotland is unnecessary for now, though as mentioned in relation to alternative approaches, if a more pathogenic strain of *M. bovis* appeared in the country that had a more significant impact on industry then this would be appropriate.

In NZ, *M. bovis* was identified in 2017, though it was believed to have arrived into the country much earlier. The disease had significant impacts on the NZ dairy industry, causing widespread mastitis within herds.

The *M. bovis* eradication programme in NZ was estimated to cost the government $722 million as of June 2024 (Dairy NZ, 2024). The eradication scheme also had a considerable impact on the mental wellbeing of farmers that had their entire herd culled.

As *M. bovis* appears to be endemic in Scotland, eradication is simply not an option as it would not be practical nor financially viable. If a more virulent strain emerges in Scotland, then eradication may be considered in the future.

Summary

It is evident that *M. bovis* is present in most dairy herds in Scotland. Until such a time that there is further research into the within-herd impacts of *M. bovis* in Scotland, the most viable option is to continue as usual. This does not mean that further testing would not be beneficial to the industry, and specifically to individual herds where the disease is a problem. BTM testing 3-4 times each year

would be a good addition to a herd's health plan along with screening youngstock.

Two vaccines are now available in the UK, and these should be utilised where possible to prevent the risk of introduction to naïve herds and youngstock rearing facilities along with good biosecurity and biocontainment practices.

Ultimately, one of the main drivers to implement future control or monitoring strategies is those within the industry. When the work for this thesis commenced, *M. bovis* was a 'hot topic' in the Scottish dairy industry, with popular farming and veterinary publications frequently including articles on the disease and was on the forefront of many people's minds. Now in 2024, *M. bovis* is not discussed nearly as much as it was in 2020 and 2021. This doesn't mean that the disease should be forgotten, but it could suggest that it may not be significantly impacting most farms, or that farmers and vets are managing the disease using their own testing regime and incorporating vaccination with some success. Other issues are higher priorities.

It's not possible to say whether farmers would be onboard with a new *M. bovis* strategy involving routine testing. Therefore, a next step would be to conduct surveys or other qualitative studies on farmer behaviour to investigate how they feel about *M. bovis* and whether they would welcome an *M. bovis* control strategy. Other research in *M. bovis* should also focus on the within-herd prevalence, quantifying how important the various transmission routes are, and understanding the financial and productivity impacts of the disease within Scottish dairy herds, as these are currently the main knowledge gaps for the disease.

Once the true impact of *M. bovis* in Scottish dairy herds is known, the industry will be better equipped to determine the most appropriate actions to tackle the disease.

Appendix 1

Figure A1-1 Flyer to recruit farms to the bulk tank milk study before COVID-19 pandemic (recruitment 1)

Will you help stop the rise of *Mycoplasma bovis*?

SRUC

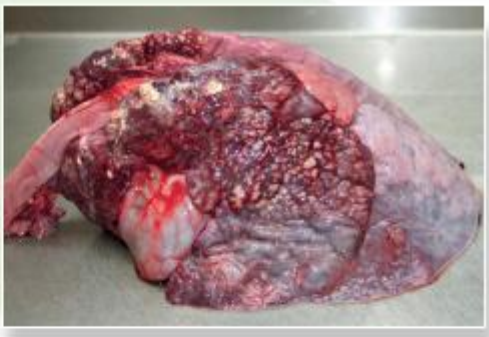
Mycoplasma bovis is an infectious pathogen in cattle that causes a range of clinical signs which have significant economic and welfare impacts. Symptoms of *M. bovis* include pneumonia and a head tilt in young stock, and mastitis and arthritis in adult cattle. Treatment options are limited and no commercial vaccines are available. We are concerned that this disease is becoming more common in Scotland.

SRUC is running a project to determine the presence of *M. bovis* across the Scottish dairy industry. The information collected will help develop more structured control plans for this disease to limit spread between and within herds, help manage the welfare and economic effects and reduce the reliance on antimicrobials.

Participants will be asked to:

- Complete a simple questionnaire on farm structure and management
- Submit 4x bulk tank milk samples over a period of 1 year with a submission form

All testing is free of charge. We will report your own herd status to you and your registered veterinary practice.



Diseased calf lung with *Mycoplasma bovis*

If you would be interested in participating in this project, please get back to us with your farm name and address so we can send you more details via:
Email: mycoplasmabovis@sruc.ac.uk
Text: 07785382371
Or the attached return slip.

Thank you

Jessica Ireland-Hughes and Colin Mason (SRUC)

University of Glasgow

Scotland's Rural College
Leading the way in Agriculture and Rural Research, Education and Consulting

419109AD 21.1.2020

Figure A1-2 Flyer to recruit farms to the bulk tank milk study after initial COVID-19 pandemic (recruitment 2)

Last call for farmers to to get involved in *Mycoplasma bovis* Project

SRUC

Mycoplasma bovis is an infectious pathogen in cattle that causes a range of clinical signs which have significant economic and welfare impacts.

Symptoms of *M. bovis* include pneumonia and a head tilt in young stock, and mastitis and lameness in adult cattle, although some cattle may show no symptoms and still spread the disease.

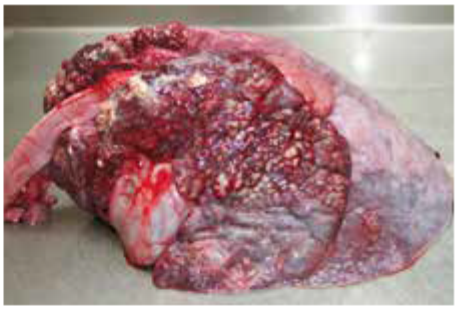
Treatment options are limited, and no commercial vaccines are available.

We are concerned that this disease is becoming more common in Scotland.

SRUC is currently running a project to determine the presence of *M. bovis* across the Scottish dairy industry.

The information collected will help develop more structured control plans for this disease to limit spread between and within herds, help manage the welfare and economic effects and reduce the reliance on antimicrobials.

We need both positive and negative herds to identify why some herds are more at risk than others.



Diseased calf lung with *Mycoplasma bovis*

Participants will be asked to:

- Complete a simple questionnaire on farm structure and management
- Submit 4x bulk tank milk samples over a period of 1year with a submission form

All testing is free of charge

We will report your own herd status to you and your registered veterinary practice.

If you are interested in participating in the project, contact us via email: mycoplasmabovis@sruc.ac.uk or by text: 07785 382371 by 30th November

University of Glasgow

Scotland's Rural College
At the Heart of the Natural Economy

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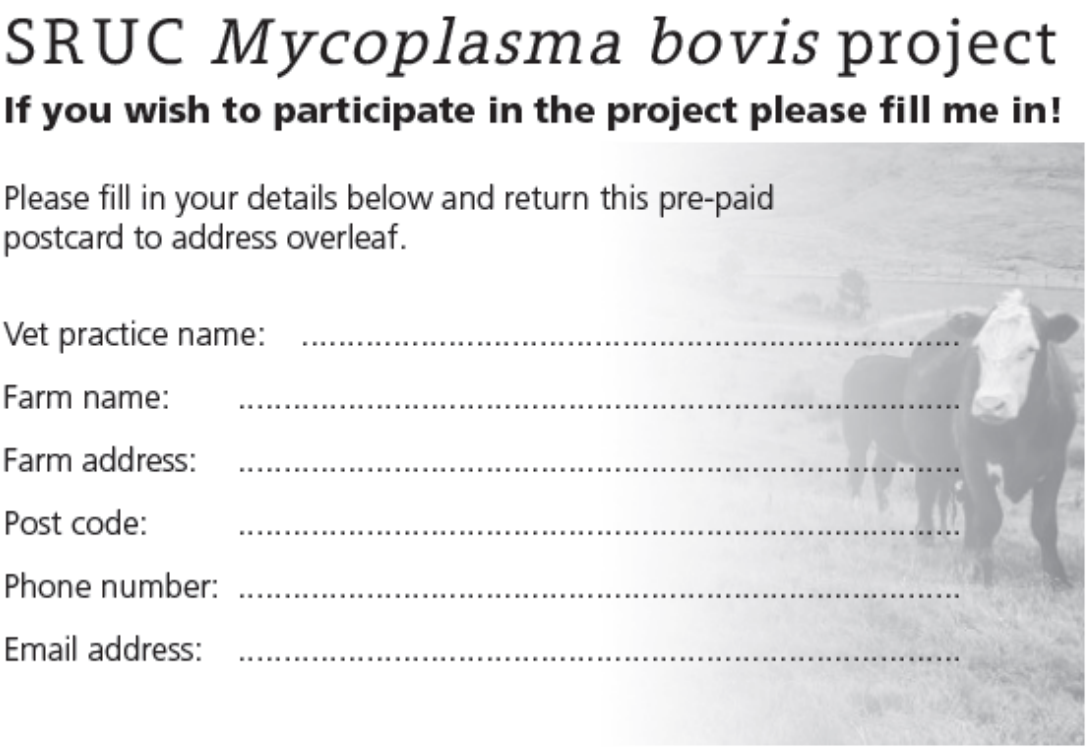
Figure A1-3 Pre-paid return slip for farmers to return if they wished to participate in the bulk tank milk study



Appendix 2

Figure A2-1 BTM study recruitment letter

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SRUC

SRUC Veterinary Services
St Mary's Industrial Estate
Dumfries DG1 1DX
Scotland UK

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www.sruc.ac.uk

12th March 2021

SRUC MYCOPLASMA BOVIS PROJECT

Thank you for volunteering to participate in the *Mycoplasma bovis* project.

Samples will be collected on three occasions (March, June and September). The samples will be tested for the presence of the *Mycoplasma bovis* pathogen and antibodies against it. Testing of these samples will be at no cost to you. We aim to batch milk samples from various farms for testing and expect the laboratory results back reasonably quickly and can report them to you and your registered veterinary practice.

Please return your sampling kit **as soon as possible**, the latest date we can receive samples is **31st March** so please send them in before this date.

Could you please read the **participant information sheet** and if you are happy to continue with the project, **sign the consent form**, fill out the **questionnaire and submission form** and return them with the bulk milk samples. We cannot process the samples or questionnaire results unless you consent.

Enclosed are 2 sample tubes, a plastic zip lock bag and prepaid postage envelope for you to return the bulk milk samples to us.

Please ensure that the milk is agitated prior to sample collection. The two sample tubes already contain a preservative tablet so do not dip them directly into the bulk tank. Fill each tube with milk almost full. Please use the zip lock back provided to prevent the milk samples leaking onto the forms.

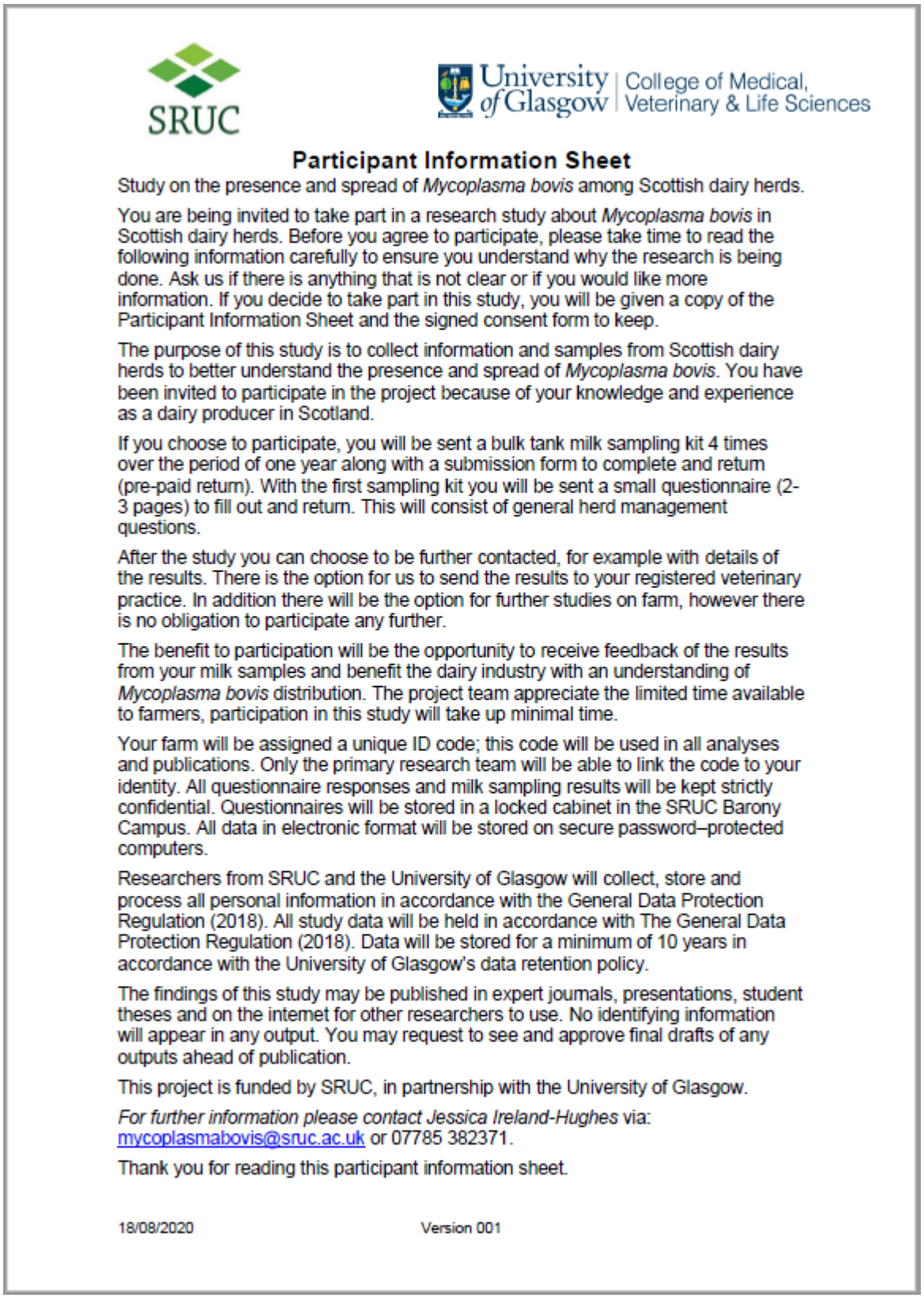
With the samplings in Jun and Sep, you will only be required to submit two bulk milk samples and complete and return a submission form.

Thank you for your help with this project, which will benefit your enterprise and the wider livestock farming community in Scotland.

Yours sincerely

Jessica Ireland-Hughes
SRUC PhD Student

Figure A2-2 BTM study participant information sheet



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Participant Information Sheet

Study on the presence and spread of *Mycoplasma bovis* among Scottish dairy herds.

You are being invited to take part in a research study about *Mycoplasma bovis* in Scottish dairy herds. Before you agree to participate, please take time to read the following information carefully to ensure you understand why the research is being done. Ask us if there is anything that is not clear or if you would like more information. If you decide to take part in this study, you will be given a copy of the Participant Information Sheet and the signed consent form to keep.

The purpose of this study is to collect information and samples from Scottish dairy herds to better understand the presence and spread of *Mycoplasma bovis*. You have been invited to participate in the project because of your knowledge and experience as a dairy producer in Scotland.

If you choose to participate, you will be sent a bulk tank milk sampling kit 4 times over the period of one year along with a submission form to complete and return (pre-paid return). With the first sampling kit you will be sent a small questionnaire (2-3 pages) to fill out and return. This will consist of general herd management questions.

After the study you can choose to be further contacted, for example with details of the results. There is the option for us to send the results to your registered veterinary practice. In addition there will be the option for further studies on farm, however there is no obligation to participate any further.

The benefit to participation will be the opportunity to receive feedback of the results from your milk samples and benefit the dairy industry with an understanding of *Mycoplasma bovis* distribution. The project team appreciate the limited time available to farmers, participation in this study will take up minimal time.

Your farm will be assigned a unique ID code; this code will be used in all analyses and publications. Only the primary research team will be able to link the code to your identity. All questionnaire responses and milk sampling results will be kept strictly confidential. Questionnaires will be stored in a locked cabinet in the SRUC Barony Campus. All data in electronic format will be stored on secure password–protected computers.

Researchers from SRUC and the University of Glasgow will collect, store and process all personal information in accordance with the General Data Protection Regulation (2018). All study data will be held in accordance with The General Data Protection Regulation (2018). Data will be stored for a minimum of 10 years in accordance with the University of Glasgow's data retention policy.

The findings of this study may be published in expert journals, presentations, student theses and on the internet for other researchers to use. No identifying information will appear in any output. You may request to see and approve final drafts of any outputs ahead of publication.

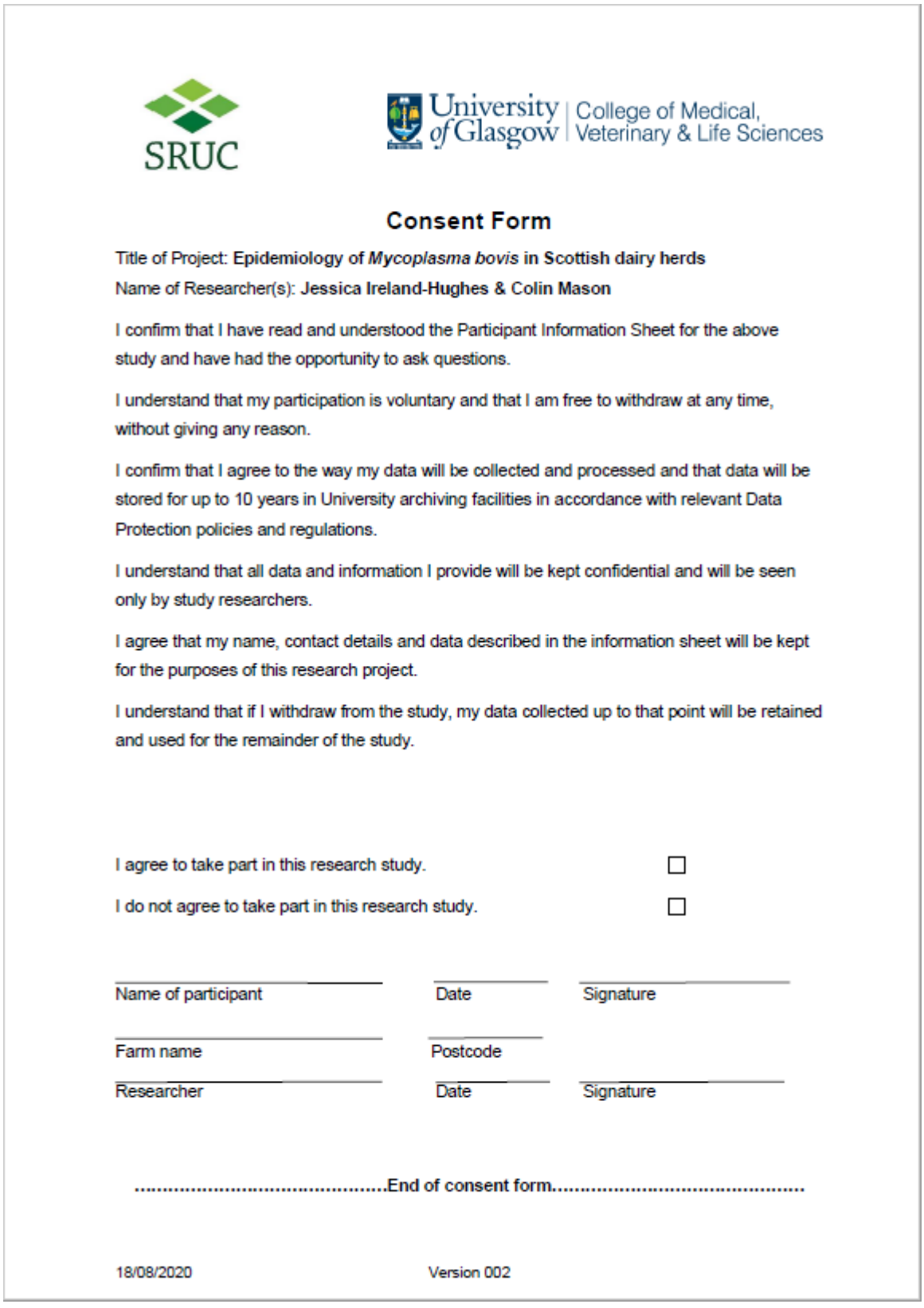
This project is funded by SRUC, in partnership with the University of Glasgow.

For further information please contact Jessica Ireland-Hughes via: mycoplasmabovis@sruc.ac.uk or 07785 382371.

Thank you for reading this participant information sheet.

18/08/2020 Version 001

Figure A2-3 BTM study consent form



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Consent Form

Title of Project: **Epidemiology of *Mycoplasma bovis* in Scottish dairy herds**

Name of Researcher(s): **Jessica Ireland-Hughes & Colin Mason**

I confirm that I have read and understood the Participant Information Sheet for the above study and have had the opportunity to ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason.

I confirm that I agree to the way my data will be collected and processed and that data will be stored for up to 10 years in University archiving facilities in accordance with relevant Data Protection policies and regulations.

I understand that all data and information I provide will be kept confidential and will be seen only by study researchers.

I agree that my name, contact details and data described in the information sheet will be kept for the purposes of this research project.

I understand that if I withdraw from the study, my data collected up to that point will be retained and used for the remainder of the study.

I agree to take part in this research study. ☐

I do not agree to take part in this research study. ☐

Name of participant _____ Date _____ Signature _____

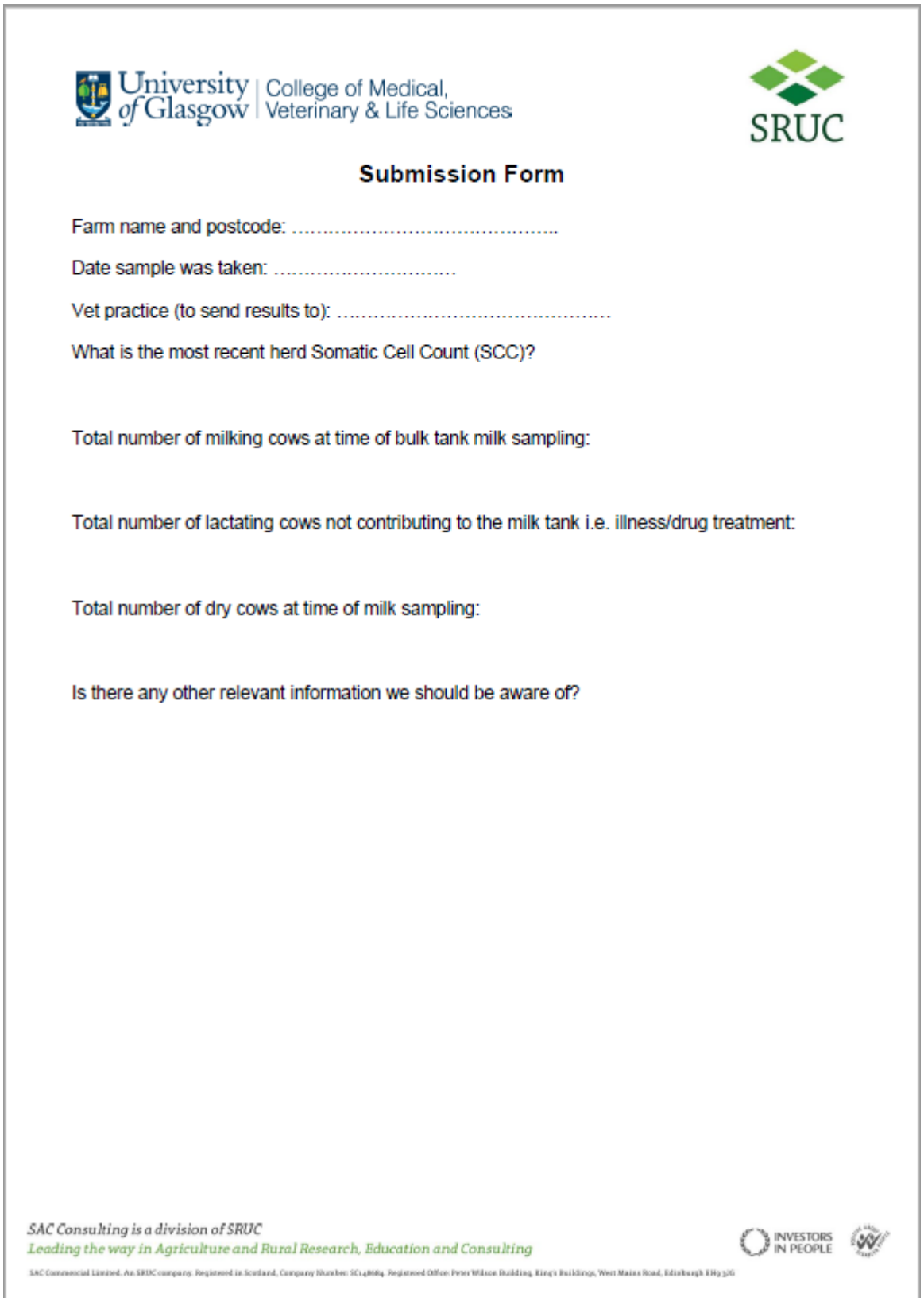
Farm name _____ Postcode _____

Researcher _____ Date _____ Signature _____

.....End of consent form.....

18/08/2020 Version 002

Figure A2-4 BTM study sample submission form



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Submission Form

Farm name and postcode: ……..

Date sample was taken: ……

Vet practice (to send results to): ……

What is the most recent herd Somatic Cell Count (SCC)?

Total number of milking cows at time of bulk tank milk sampling:

Total number of lactating cows not contributing to the milk tank i.e. illness/drug treatment:

Total number of dry cows at time of milk sampling:

Is there any other relevant information we should be aware of?

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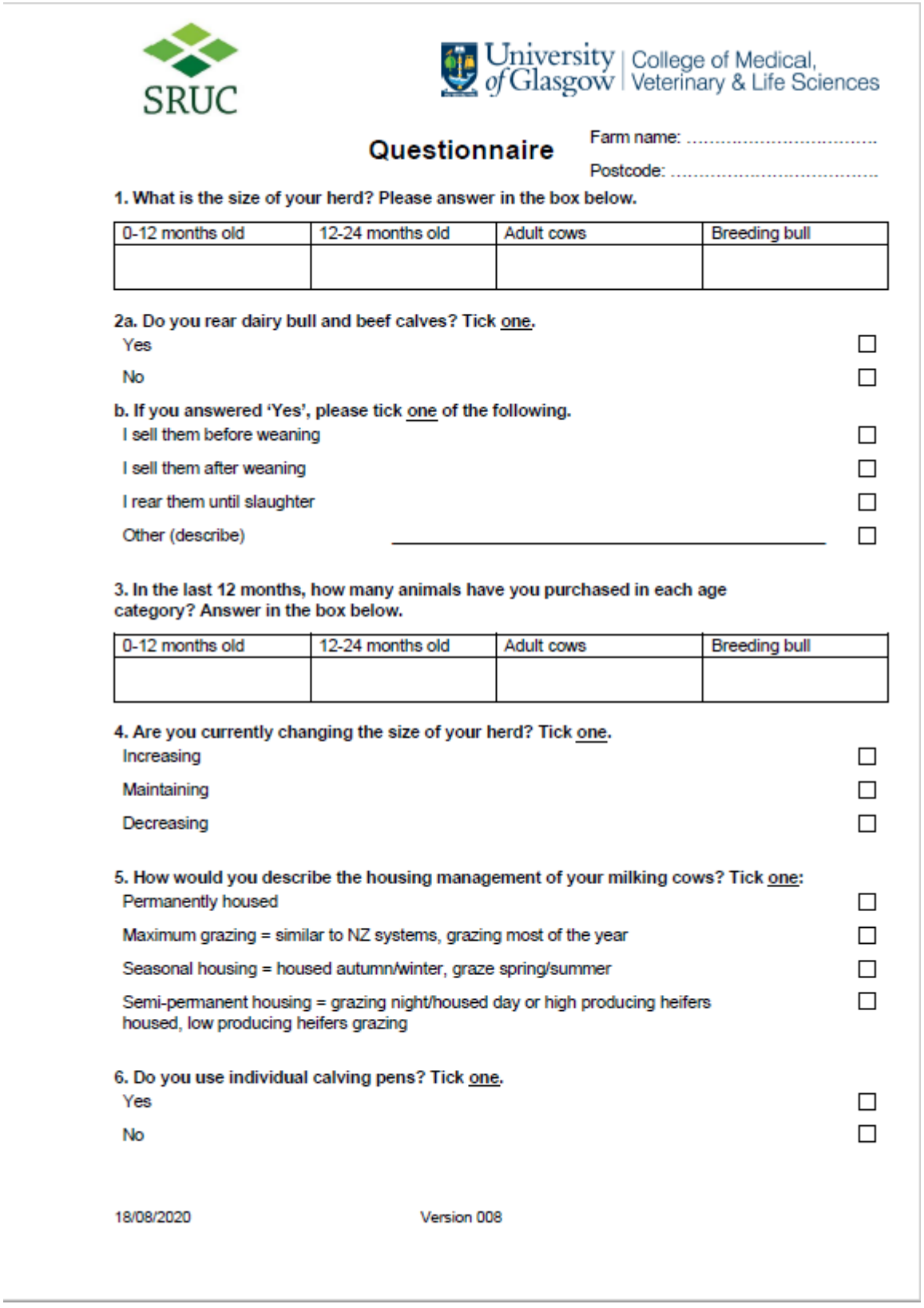
Figure A2-5 BTM study questionnaire



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Questionnaire

Farm name: .....

Postcode: .....

1. What is the size of your herd? Please answer in the box below.

0-12 months old	12-24 months old	Adult cows	Breeding bull

2a. Do you rear dairy bull and beef calves? Tick one.

Yes ☐

No ☐

b. If you answered 'Yes', please tick one of the following.

I sell them before weaning ☐

I sell them after weaning ☐

I rear them until slaughter ☐

Other (describe) _____ ☐

3. In the last 12 months, how many animals have you purchased in each age category? Answer in the box below.

0-12 months old	12-24 months old	Adult cows	Breeding bull

4. Are you currently changing the size of your herd? Tick one.

Increasing ☐

Maintaining ☐

Decreasing ☐

5. How would you describe the housing management of your milking cows? Tick one:

Permanently housed ☐

Maximum grazing = similar to NZ systems, grazing most of the year ☐

Seasonal housing = housed autumn/winter, graze spring/summer ☐

Semi-permanent housing = grazing night/housed day or high producing heifers housed, low producing heifers grazing ☐

6. Do you use individual calving pens? Tick one.

Yes ☐

No ☐

18/08/2020 Version 008

7a. What calving pattern do you operate? Tick one.

Year-round calving ☐

Block calving ☐

b. If block calving, tick all that apply.

Spring ☐

Summer ☐

Autumn ☐

Winter ☐

8. Housing of milk-fed, pre-weaned calves. Tick all that apply.

Individually housed then group fed ☐

Group housing ☐

Individually housed until weaning ☐

Other (describe) _____ ☐

9. Housing of post-weaned calves. Tick all that apply.

Grouped without mixing – remain in same group from weaning ☐

Grouped with mixing – do not remain in same group ☐

Other (describe) _____ ☐

10a. Describe your colostrum feeding policy. Tick one.

Artificial ☐

Cows colostrum ☐

Both ☐

b. If you feed cow's colostrum, do you pasteurise it? Tick one.

Yes ☐

No ☐

11a. Describe your milk feeding policy. Tick one.

Milk replacer ☐

Whole milk ☐

Both ☐

b. If you feed cow's milk, do you pasteurise it? Tick one.

Yes ☐

No ☐

12. How is milk given to calves up to weaning? Tick all that apply.

- Individual bucket ☐
- Individual bucket and teat ☐
- Group feeding teat ☐
- Automatic feeder ☐
- Other (describe) _____ ☐

13. How often do you clean and disinfect calf feeding utensils? Tick all that apply.

- In between each calf ☐
- Between batches of calves ☐
- Once daily ☐
- Twice daily ☐
- After every feeding session ☐
- Other (describe) _____ ☐

14. Were you aware of *M. bovis* before participating in this project? Tick one.

- I was not aware of *Mycoplasma bovis* ☐
- I was aware of *Mycoplasma bovis* ☐

15. Is *M. bovis* present in your herd? Tick all that apply.

- I previously had *Mycoplasma bovis* in my herd, confirmed diagnosis by vet ☐
- To my knowledge, *Mycoplasma bovis* has never been present in my herd ☐
- I and/or my vet currently/previously suspect that *Mycoplasma bovis* is in my herd (no confirmed diagnosis) ☐
- I currently have *Mycoplasma bovis* in my herd, confirmed diagnosis by vet ☐
- I have never considered that *Mycoplasma bovis* could be in my herd ☐

16. Do you use a vaccine against *M. bovis*? Tick one.

- Yes (describe): _____ ☐
- No ☐

Appendix 3

Figure A3-1 *Mycoplasma bovis* factsheet 1. Providing information to farmers with their results as a thank you for participating

FACTSHEET 1/3. The aim of these factsheets is to help inform participating farmers that have been good enough to help us with the project about *M. bovis*. We hope this is of interest to you and if you have any questions about the information feel free to get in touch!

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What is *Mycoplasma bovis*?

M. bovis is an infectious pathogen that causes a variety of disease symptoms in all ages of cattle.

It is not enclosed in a cell wall. This is very important as:

- The most commonly used antimicrobials (e.g. penicillin) are ineffective against *M. bovis* as they target the cell wall.
- *M. bovis* can change its shape and size in different environments which may help the pathogen hide from the cow's immune system.

Another interesting feature is that *M. bovis* creates a biofilm which is a protective layer that also helps it evade the immune system.

This is what *M. bovis* looks like under a microscope. It is described as having a 'fried egg' appearance!

Image from: Karahan *et al.* (2010), http://dx.doi.org/10.1136/vr.b4864

What *M. bovis* looks like in calves
Pneumonia: cough, abnormal nasal discharge fever, depression, reduced appetite
Ear disease: head tilt, one or both ear droop
Arthritis/synovitis: lame on one or multiple legs, swollen joints

What *M. bovis* looks like in adult cattle
Mastitis: sudden drop in milk production, foul pus secreted from one or more quarters, firm quarters
Arthritis/synovitis: lame on one or multiple legs, swollen joints
Eye disease: aversion to light, discharge, eye spasms, watery eyes, inability to locate food, blindness
Reproductive disease: abortions, infertility

Not all cows & calves develop symptoms when exposed but can still infect the rest of the herd!

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Figure A3-2 *Mycoplasma bovis* factsheet 2. Providing information to farmers with their results as a thank you for participating

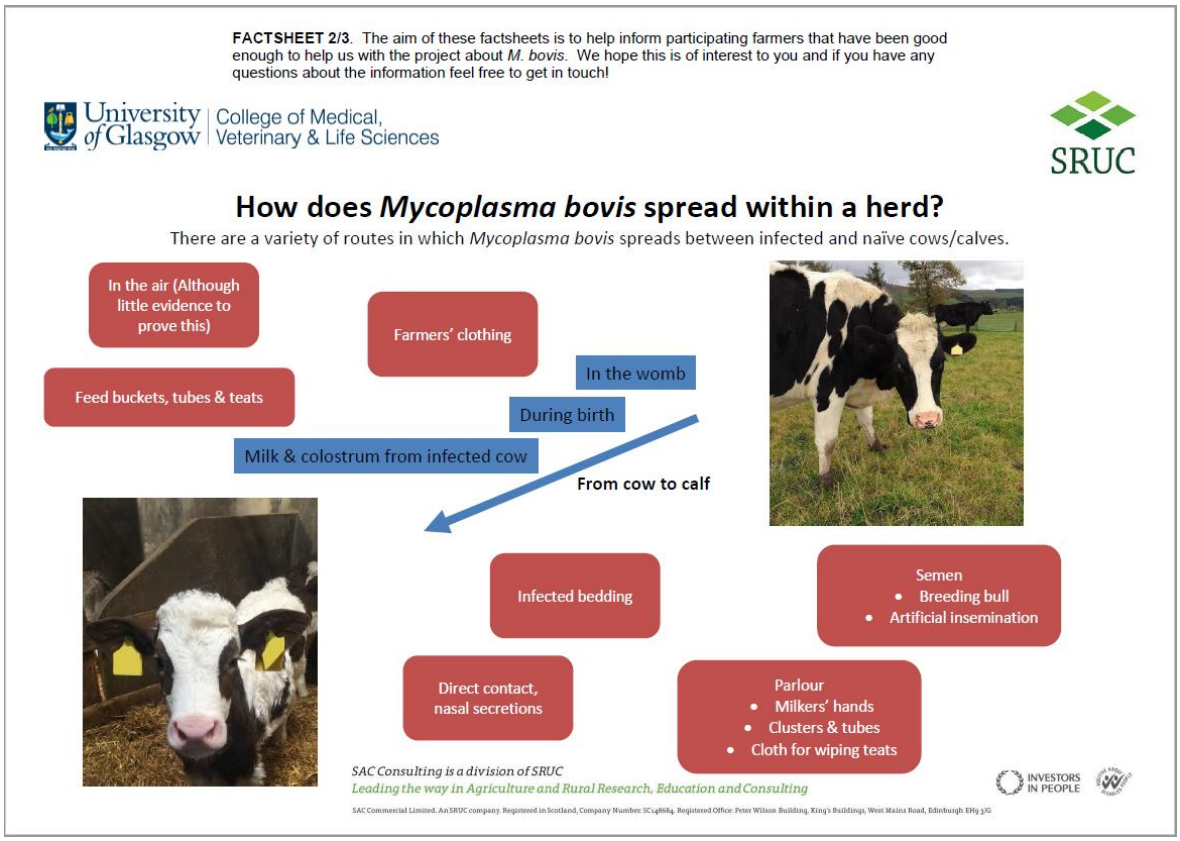

Figure A3-3 *Mycoplasma bovis* factsheet 3. Providing information to farmers with their results as a thank you for participating

FACTSHEET 3/3. The aim of these factsheets is to help inform participating farmers that have been good enough to help us with the project about *M. bovis*. We hope this is of interest to you and if you have any questions about the information feel free to get in touch!

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Reducing the risk of *Mycoplasma bovis* in your herd

Ask yourself, what do you think your current risk of *M. bovis* is? Is it present in your herd *and* causing a problem?

The testing you have been doing in this *M. bovis* study will help you to assess the risk on your farm.

The impact of *M. bovis* is different on every farm, so a good way to tell if it may be having an impact on your productivity is benchmarking calf performance:

- Pneumonia treatment rates
- Growth rates

Are the treatment rates very high? Are the growth rates relatively low? Could they be better?

Also, *M. bovis* has been shown to reduce milk production by 3kg/cow/day.

Based on current thinking, there are a number of ways you can reduce the risk of *M. bovis* in your herd.

Parlour hygiene:

- Regularly clean & disinfect
- If forestripping – use separate cloths to wipe each cow's teat

Avoid buying in breeding replacements or a breeding bull.

Have a separate calving box/pen from the main herd.

Reduce group size of calves as much as possible & try to narrow the age range within each group, i.e. avoid having 1-week old calves in a group with 6-month-old calves.

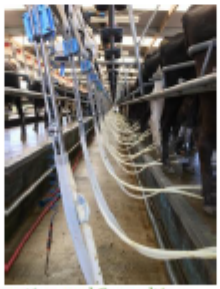
Calf feeding

Feeding milk & colostrum from cows to calves are a key route of spreading *M. bovis*. Some things you may want to consider:

- Move to calf milk replacer rather than cows milk ASAP
- Pasteurise milk and colostrum
- Avoid feeding waste milk

M. bovis survives for long periods of time in the environment:

- Maintain calf feeding equipment (damage can harbour bacteria incl. *M. bovis*)
- Regularly clean & disinfect feeding equipment



Please note
It is always best practice to discuss any concerns you may have with your vet.

Also, not all changes may be appropriate to your system, but with the help from your vet you can identify what changes you can make.

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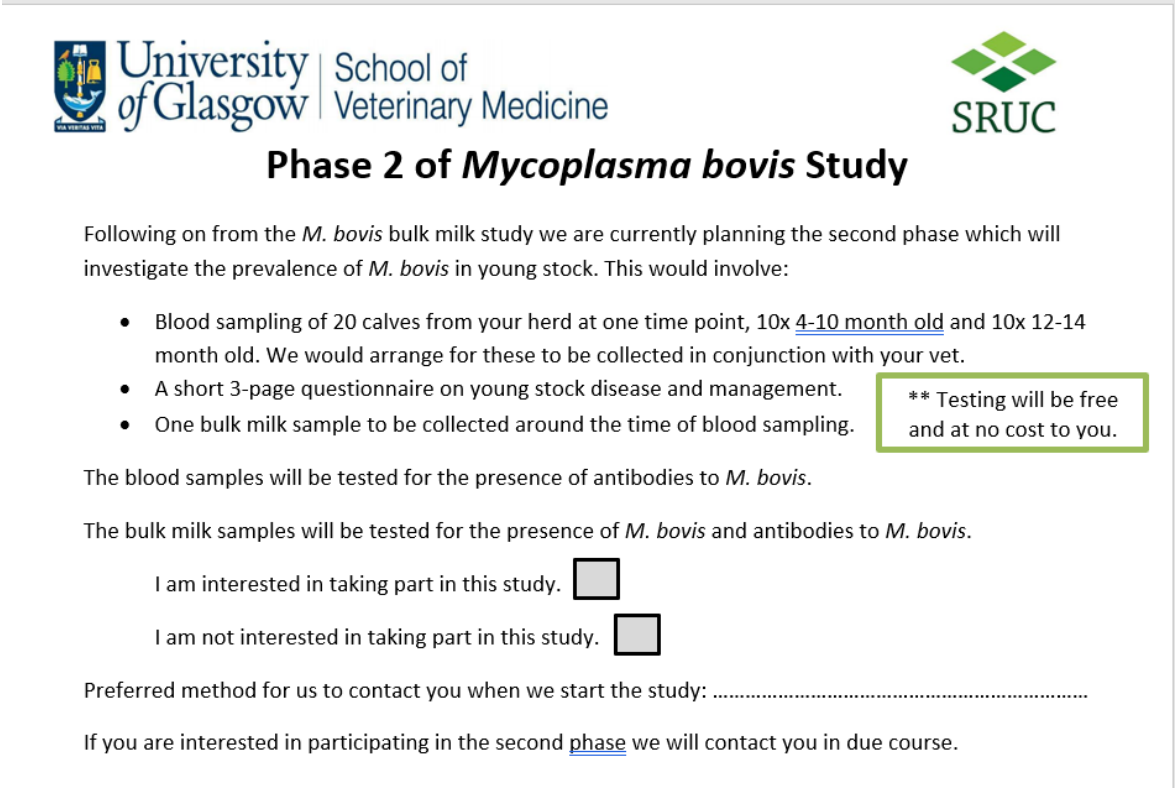
Appendix 4

Figure A4-1 Interest slip for the calf study sent to farmers at the end of the BTM study



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Phase 2 of *Mycoplasma bovis* Study

Following on from the *M. bovis* bulk milk study we are currently planning the second phase which will investigate the prevalence of *M. bovis* in young stock. This would involve:

- Blood sampling of 20 calves from your herd at one time point, 10x 4-10 month old and 10x 12-14 month old. We would arrange for these to be collected in conjunction with your vet.
- A short 3-page questionnaire on young stock disease and management.
- One bulk milk sample to be collected around the time of blood sampling.

** Testing will be free and at no cost to you.

The blood samples will be tested for the presence of antibodies to *M. bovis*.

The bulk milk samples will be tested for the presence of *M. bovis* and antibodies to *M. bovis*.

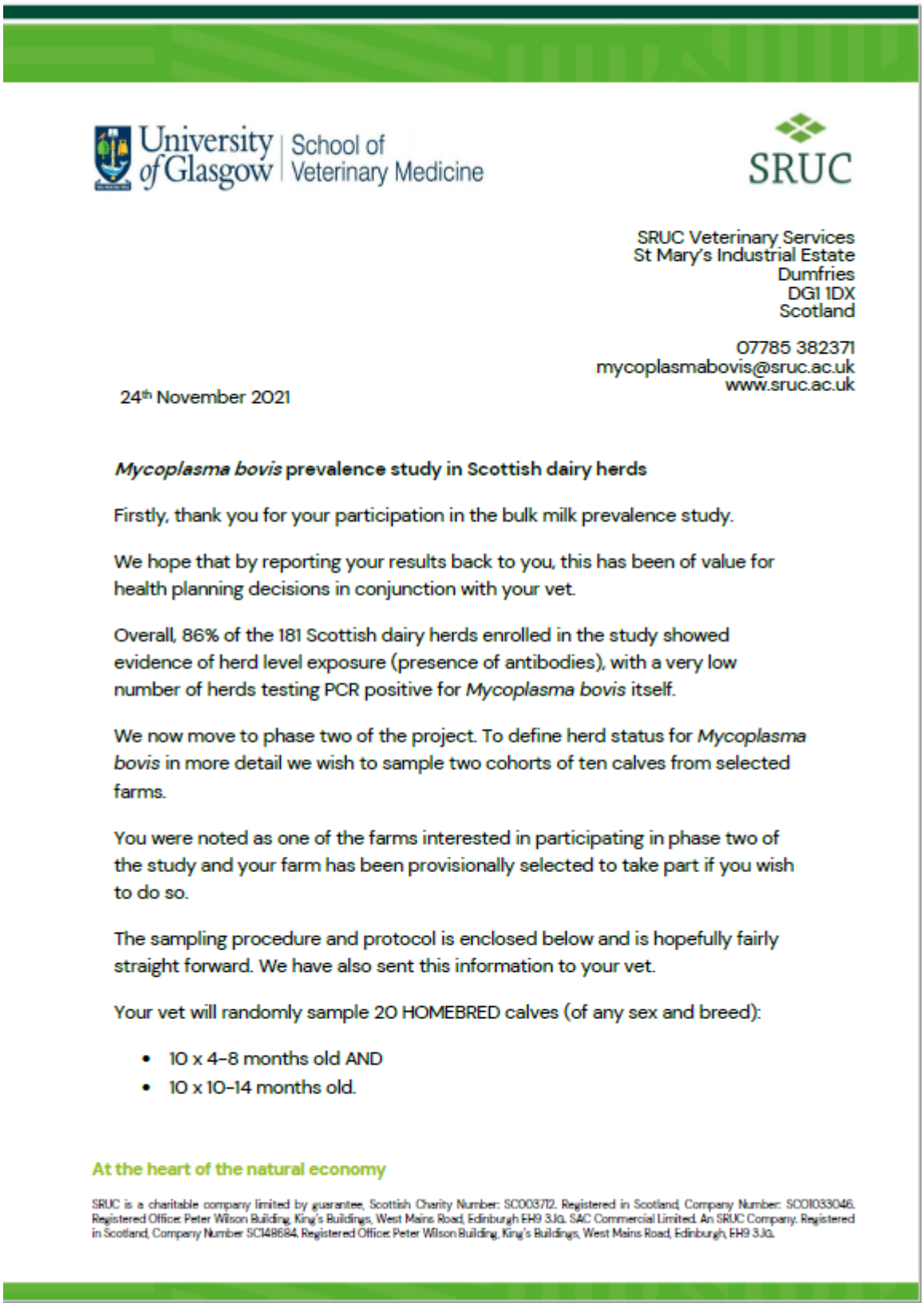
I am interested in taking part in this study. ☐

I am not interested in taking part in this study. ☐

Preferred method for us to contact you when we start the study: .....

If you are interested in participating in the second phase we will contact you in due course.

Figure A4-2 Recruitment letter sent to farmers for calf study



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St Mary's Industrial Estate
Dumfries
DG1 1DX
Scotland

07785 382371
mycoplasmabovis@sruc.ac.uk
www.sruc.ac.uk

24th November 2021

***Mycoplasma bovis* prevalence study in Scottish dairy herds**

Firstly, thank you for your participation in the bulk milk prevalence study.

We hope that by reporting your results back to you, this has been of value for health planning decisions in conjunction with your vet.

Overall, 86% of the 181 Scottish dairy herds enrolled in the study showed evidence of herd level exposure (presence of antibodies), with a very low number of herds testing PCR positive for *Mycoplasma bovis* itself.

We now move to phase two of the project. To define herd status for *Mycoplasma bovis* in more detail we wish to sample two cohorts of ten calves from selected farms.

You were noted as one of the farms interested in participating in phase two of the study and your farm has been provisionally selected to take part if you wish to do so.

The sampling procedure and protocol is enclosed below and is hopefully fairly straight forward. We have also sent this information to your vet.

Your vet will randomly sample 20 HOMEBRED calves (of any sex and breed):

- 10 x 4–8 months old AND
- 10 x 10–14 months old.

As we are testing for antibodies, if you use a *Mycoplasma bovis* vaccine then unfortunately you cannot participate in the study. If between registering for the study and sampling you introduce an *M. bovis* vaccine, please let us know.

We also ask if you could please collect a bulk milk sample on the same day in which your vet samples the calves. There is also a short questionnaire on calf husbandry to be completed.

We will supply all sampling materials and all testing will be free of charge.

We hope that you will find it possible to sample the youngstock during this winter housing period, and that this could be incorporated into a planned visit from your vet. We hope that in return that the results and information will be of real use to you.

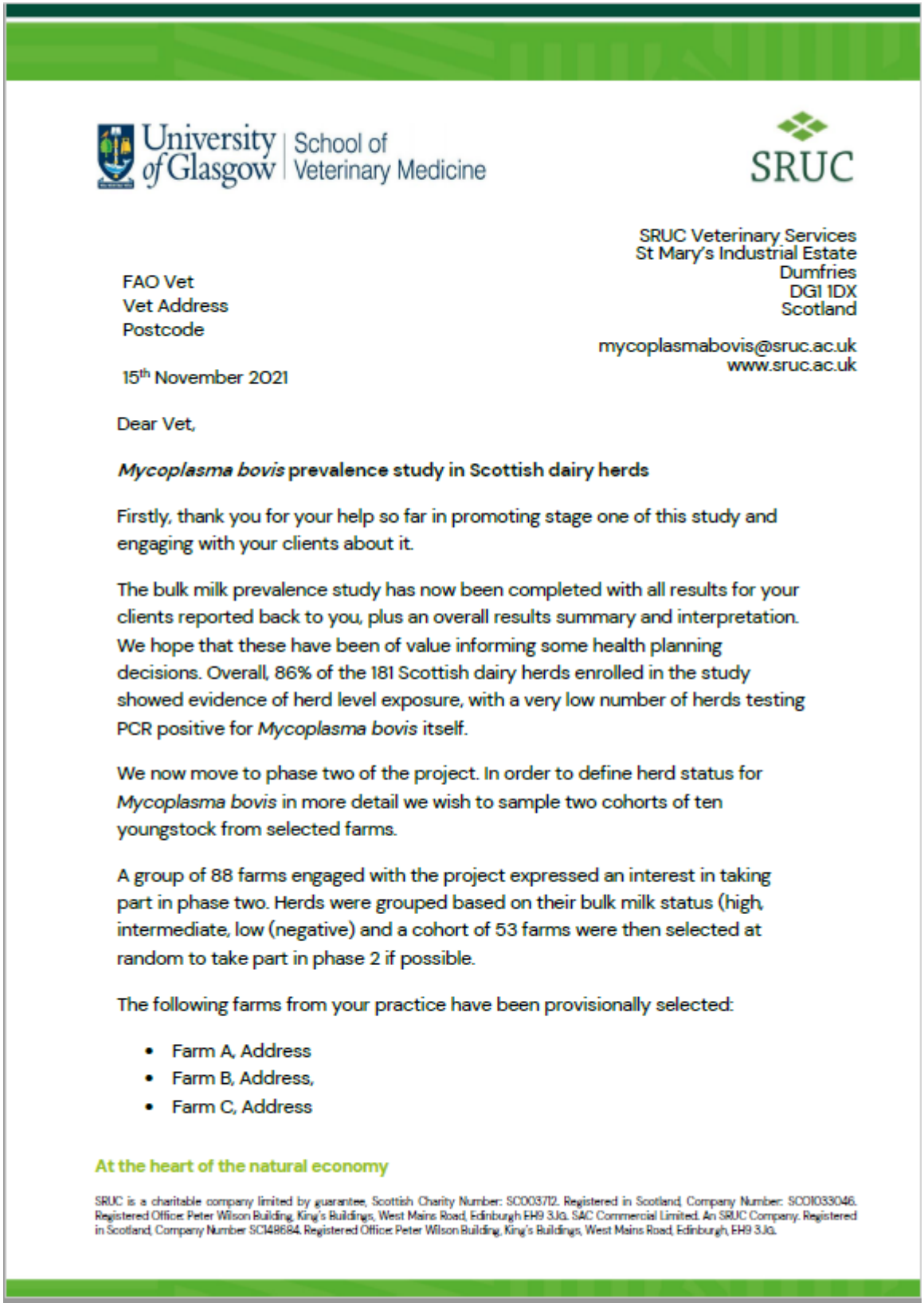
Overall, the information generated collectively by this project will help inform future control strategies for this important disease.

If you wish to participate, please respond either by email, phone, or post (contact details above), and if you have any questions or concerns, please do not hesitate to get in touch.

Yours sincerely,

Colin Mason and Jessica Ireland-Hughes

Figure A4-3 Recruitment letter sent to vets for calf study



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SRUC Veterinary Services
St Mary's Industrial Estate
Dumfries
DG1 1DX
Scotland

mycoplasmabovis@sruc.ac.uk
www.sruc.ac.uk

FAO Vet
Vet Address
Postcode

15th November 2021

Dear Vet,

***Mycoplasma bovis* prevalence study in Scottish dairy herds**

Firstly, thank you for your help so far in promoting stage one of this study and engaging with your clients about it.

The bulk milk prevalence study has now been completed with all results for your clients reported back to you, plus an overall results summary and interpretation. We hope that these have been of value informing some health planning decisions. Overall, 86% of the 181 Scottish dairy herds enrolled in the study showed evidence of herd level exposure, with a very low number of herds testing PCR positive for *Mycoplasma bovis* itself.

We now move to phase two of the project. In order to define herd status for *Mycoplasma bovis* in more detail we wish to sample two cohorts of ten youngstock from selected farms.

A group of 88 farms engaged with the project expressed an interest in taking part in phase two. Herds were grouped based on their bulk milk status (high, intermediate, low (negative) and a cohort of 53 farms were then selected at random to take part in phase 2 if possible.

The following farms from your practice have been provisionally selected:

- Farm A, Address
- Farm B, Address,
- Farm C, Address

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The sampling procedure and protocol is enclosed below and is hopefully fairly straight forward.

Could you please randomly sample 20 HOMEBRED calves:

- 10 x 4–8 months old AND
- 10 x 10–14 months old.

Include calves of any sex and breed.

As we are testing for antibodies, if the farm uses a *Mycoplasma bovis* vaccine then they cannot participate in the study. If between registering for the study and sampling the farm introduces an *M. bovis* vaccine, please let us know.

Please can the farmer also collect a sample of the bulk milk.

We will supply all sampling materials and all testing will be free of charge.

We hope that you will find it possible to sample the youngstock during this winter housing period, and that this could be incorporated into a planned visit. We do not have the funding to find time for sampling and we hope that you will be able to help us with that. We hope that in return that the results and information will be of real use to your clients and your practice.

Overall, the information generated collectively by this project will help inform future control strategies for this important disease.

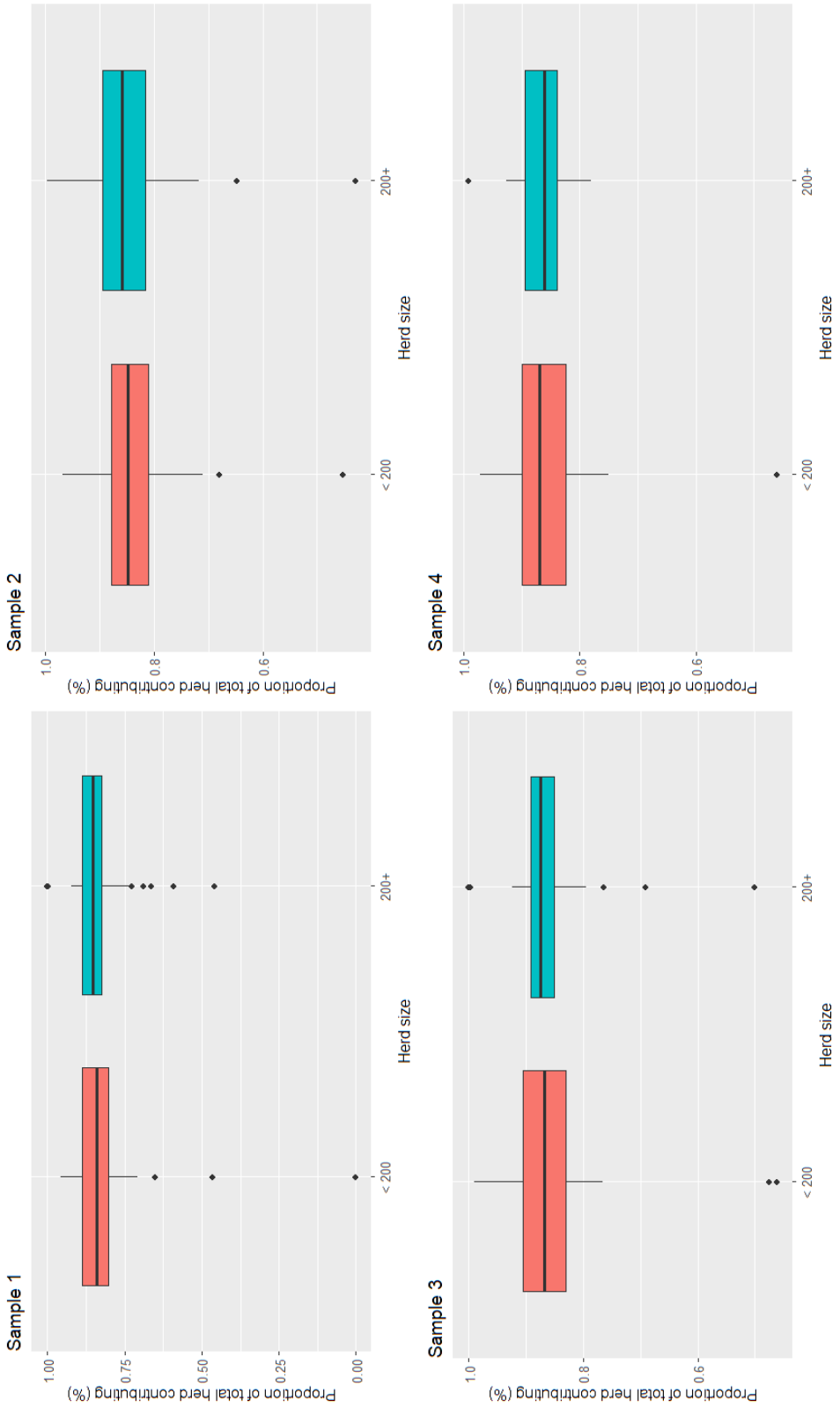
If you have any questions or concerns, please do not hesitate to get in touch with us at the above address.

Yours sincerely,

Colin Mason and Jessica Ireland-Hughes

Appendix 5

Figure A5-1 The proportion of the total herd that contributed to each BTM sample by herd size (<200, ≥200).



Appendix 6

Figure A6-1 Longitudinal BTM results of the seven farms that reported using a vaccine against *M. bovis*

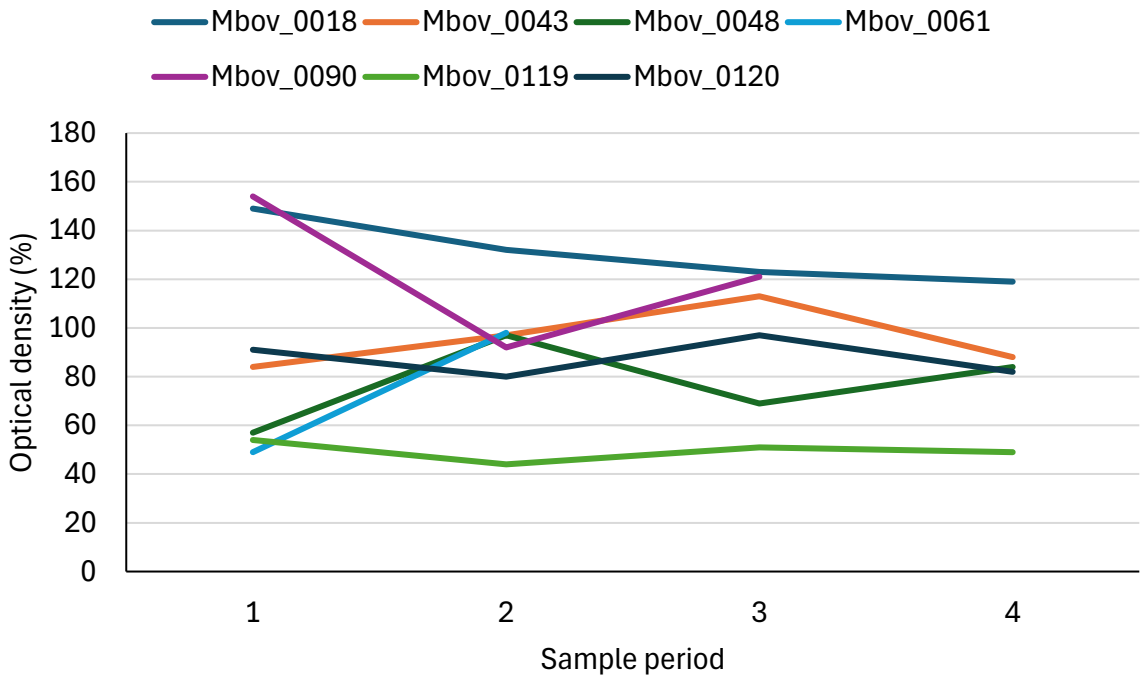


Figure A6-2 Longitudinal optical density trendlines for every herd across all four sampling points. Test cut-off of 30% is represented by the black dotted line.

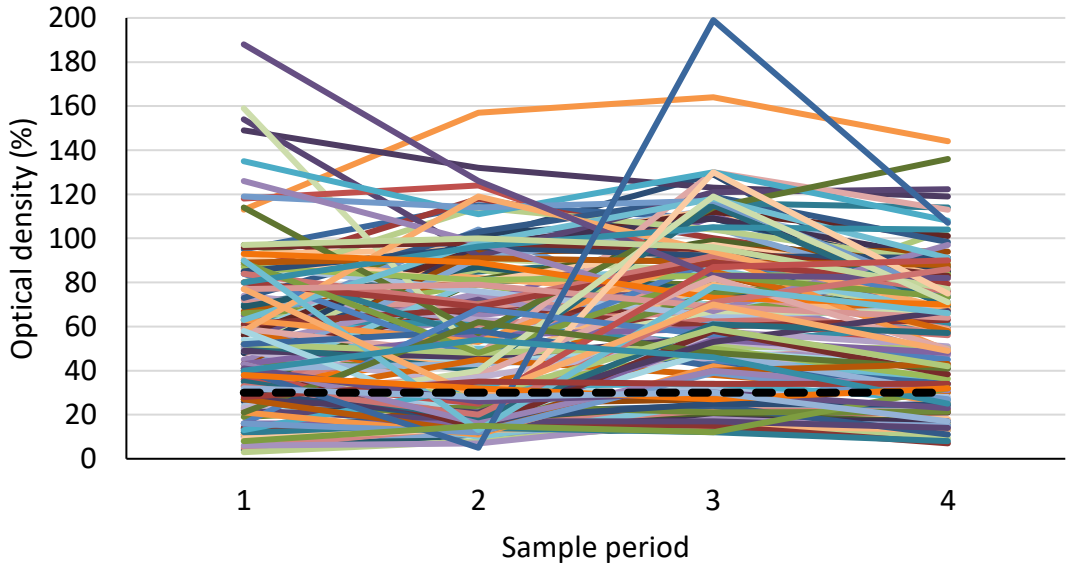


Figure to illustrate the considerable variation in the OD% trends in all study herds.

Table A6-1 Results of comparing the final model (A) and two alternative models (B and C) using the Akaike Information Criterion (AIC)

Modnames	K	AICc	Delta_AICc	ModelLik	AICcWt	LL	Cum.Wt
Model A	2	190.1894	0	1	0.9747	-93.0608	0.9747
Model B	3	198.4776	8.2882	0.0159	0.0155	-96.171	0.9901
Model C	2	199.376	9.1866	0.0101	0.0098	-97.6543	1

Appendix 7

Table A7-1 BTM Clusters – based on ELISA test cut-off. Cluster summary table

Sample no.	Value	Consistently positive	Consistently negative	Transitional	Seroconverted	Sero-reverted	Other
	N farms	81	20	32	12	2	18
	% farms	49	12	19	7	1	11
Sample 1	Min	30	3	8	8	30	13
	Max	188	27	90	29	40	90
	Range	158	24	82	21	10	77
	Mean	72.77	12.5	32.28	21.17	35	39.39
	Median	63	11.5	29.5	23	35	37
Sample 2	Min	30	7	5	12	18	13
	Max	157	25	68	68	54	33
	Range	127	18	63	56	36	20
	Mean	78.71	15.2	26.75	33.58	36	21.17
	Median	80	14.5	25.5	36	36	22.5
Sample 3	Min	42	12	12	12	25	13
	Max	164	27	199	130	46	199
	Range	122	15	187	118	21	186
	Mean	85.09	19	57.72	60	35.5	58.67
	Median	84	18	49.5	53.5	35.5	49.5
Sample 4	Min	30	7	17	30	24.33	13
	Max	144	26	107	75	25	107
	Range	114	19	90	45	0.67	94
	Mean	74.98	16.12	45.33	48.22	24.67	45.70
	Median	73.33	15	41.17	44	24.67	37.33

Table A7-2 Results of the Chi-square and Fishers exact tests to test for associations between the questionnaire responses and k-means cluster assignment

Factors	Increasing cluster	Stable cluster	Decreasing cluster	p-value
Number of 0-12 month olds				
<100	14 (41%)	25 (43%)	18 (49%)	
100+	20 (59%)	33 (57%)	19 (51%)	0.256
Number of 12-24 month olds				
<100	18 (53%)	36 (62%)	19 (51%)	
100+	16 (47%)	22 (38%)	18 (49%)	0.194
Had a breeding bull				
Yes	4 (12%)	12 (21%)	13 (35%)	
No	30 (88%)	46 (79%)	24 (65%)	*0.056
Number of cows				
<200	16 (47%)	28 (48%)	19 (51%)	
200+	18 (53%)	30 (52%)	18(49%)	0.891
Total number of cattle				
<300	7 (21%)	18 (31%)	11 (30%)	
300+	27 (79%)	40 (69%)	26 (70%)	0.488
Rear dairy bull and beef calves				
Yes	32 (91%)	51 (86%)	36 (92%)	
No	3 (9%)	8 (14%)	3 (8%)	0.631
Length of time rear dairy bull and beef calves				
Sell before weaning	6 (19%)	8 (15%)	9 (24%)	
All after weaning	25 (81%)	44 (85%)	29 (76%)	0.610
Bought in 0-12 month olds				
Yes	6 (17%)	1 (2%)	7 (18%)	
No	29 (83%)	58 (98%)	32 (82%)	*0.005
Bought in 12-24 month olds				
Yes	25 (71%)	25 (42%)	21 (54%)	
No	10 (29%)	34 (58%)	18 (46%)	*0.024
Bought in a bull				
Yes	16 (46%)	19 (32%)	13 (33%)	
No	19 (54%)	40 (68%)	26 (67%)	0.383
Bought in cow				
Yes	11 (32%)	7 (12%)	11 (28%)	
No	23 (68%)	52 (88%)	28 (72%)	*0.044
Bought in any cattle				
Yes	25 (71%)	25 (42%)	21 (54%)	
No	10 (29%)	34 (58%)	18 (46%)	*0.024

Factors	Increasing cluster	Stable cluster	Decreasing cluster	p-value
Changing herd size				
Increasing	8 (24%)	17 (29%)	11 (28%)	
Maintaining/decreasing	26 (76%)	42 (71%)	28 (72%)	0.806
Cow housing				
Permanently housed	10 (29%)	16 (27%)	13 (33%)	
Not permanently housed	24 (71%)	43 (73%)	26 (67%)	0.789
Separate calving pen				
Yes	13 (37%)	24 (41%)	8 (21%)	
No	22 (63%)	35 (59%)	31 (79%)	0.106
Pre-weaned calf housing				
Individual housing some time	26 (74%)	45 (76%)	30 (77%)	
Group housed entire life	9 (26%)	14 (24%)	9 (23%)	0.962
Post-weaned calf housing				
Don't mix	7 (21%)	17 (29%)	14 (36%)	
Mix	27 (79%)	42 (71%)	25 (64%)	0.319
Group feeding				
Individual only	8 (24%)	17 (29%)	9 (23%)	
Group only or both	26 (76%)	41 (71%)	30 (77%)	0.711
Awareness of *M. bovis*				
Not aware	7 (21%)	12 (21%)	6 (15%)	
Aware	27 (79%)	46 (79%)	33 (85%)	0.794
Herd history				
Never present	17 (50%)	30 (52%)	22 (56%)	
Suspect or confirmed	17 (50%)	28 (48%)	17 (44%)	0.792

*Statistically significant

Appendix 8

Figure A8-1 Calf study cover letter

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SRUC

SRUC Veterinary Services
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Dumfries DG1 1DX
Scotland UK

Tel: +44 (0)1387 267260
Fax: +44 (0)1387 250028
Email: vedumfries@sac.co.uk
www.sruc.ac.uk

8th July 2021

SRUC MYCOPLASMA BOVIS CALF STUDY

Thank you for your participation in the *Mycoplasma bovis* calf serology study.

The bulk milk and blood samples will be tested for the presence of antibodies to *Mycoplasma bovis*.

Enclosed are:

- 1x bulk milk sample tube
- 20x needles (+ 2 spare)
- 20x vaccutainers (+ 2 spare)
- 20x blood tubes (+ 2 spare)
- Prepaid postage envelope for you to return the blood & bulk milk samples to us.

Please ensure that the milk is agitated prior to sample collection. The sample tube already contains a preservative tablet so do not dip it directly into the bulk tank. Fill each tube with milk almost full please. Please use the zip lock back provided to prevent the milk sample leaking onto the forms.

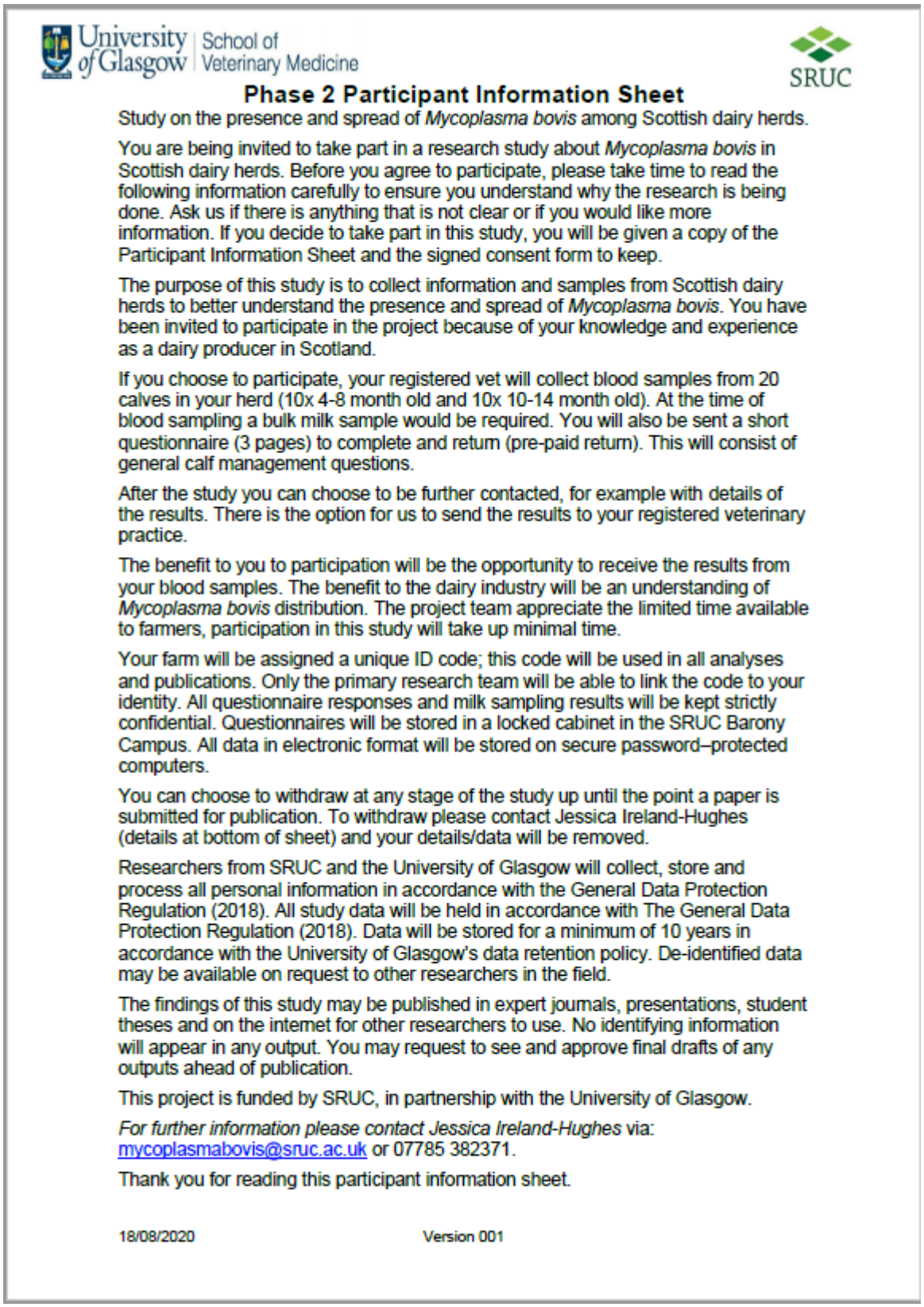
We will send out a summary of results to all participating farms.

Thank you again for your help with this project, which will benefit your enterprise and the wider livestock farming community in Scotland.

Yours sincerely

Jessica Ireland-Hughes
SRUC PhD Student

Figure A8-2 Calf study participant information sheet



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Phase 2 Participant Information Sheet

Study on the presence and spread of *Mycoplasma bovis* among Scottish dairy herds.

You are being invited to take part in a research study about *Mycoplasma bovis* in Scottish dairy herds. Before you agree to participate, please take time to read the following information carefully to ensure you understand why the research is being done. Ask us if there is anything that is not clear or if you would like more information. If you decide to take part in this study, you will be given a copy of the Participant Information Sheet and the signed consent form to keep.

The purpose of this study is to collect information and samples from Scottish dairy herds to better understand the presence and spread of *Mycoplasma bovis*. You have been invited to participate in the project because of your knowledge and experience as a dairy producer in Scotland.

If you choose to participate, your registered vet will collect blood samples from 20 calves in your herd (10x 4-8 month old and 10x 10-14 month old). At the time of blood sampling a bulk milk sample would be required. You will also be sent a short questionnaire (3 pages) to complete and return (pre-paid return). This will consist of general calf management questions.

After the study you can choose to be further contacted, for example with details of the results. There is the option for us to send the results to your registered veterinary practice.

The benefit to you to participation will be the opportunity to receive the results from your blood samples. The benefit to the dairy industry will be an understanding of *Mycoplasma bovis* distribution. The project team appreciate the limited time available to farmers, participation in this study will take up minimal time.

Your farm will be assigned a unique ID code; this code will be used in all analyses and publications. Only the primary research team will be able to link the code to your identity. All questionnaire responses and milk sampling results will be kept strictly confidential. Questionnaires will be stored in a locked cabinet in the SRUC Barony Campus. All data in electronic format will be stored on secure password–protected computers.

You can choose to withdraw at any stage of the study up until the point a paper is submitted for publication. To withdraw please contact Jessica Ireland-Hughes (details at bottom of sheet) and your details/data will be removed.

Researchers from SRUC and the University of Glasgow will collect, store and process all personal information in accordance with the General Data Protection Regulation (2018). All study data will be held in accordance with The General Data Protection Regulation (2018). Data will be stored for a minimum of 10 years in accordance with the University of Glasgow's data retention policy. De-identified data may be available on request to other researchers in the field.

The findings of this study may be published in expert journals, presentations, student theses and on the internet for other researchers to use. No identifying information will appear in any output. You may request to see and approve final drafts of any outputs ahead of publication.

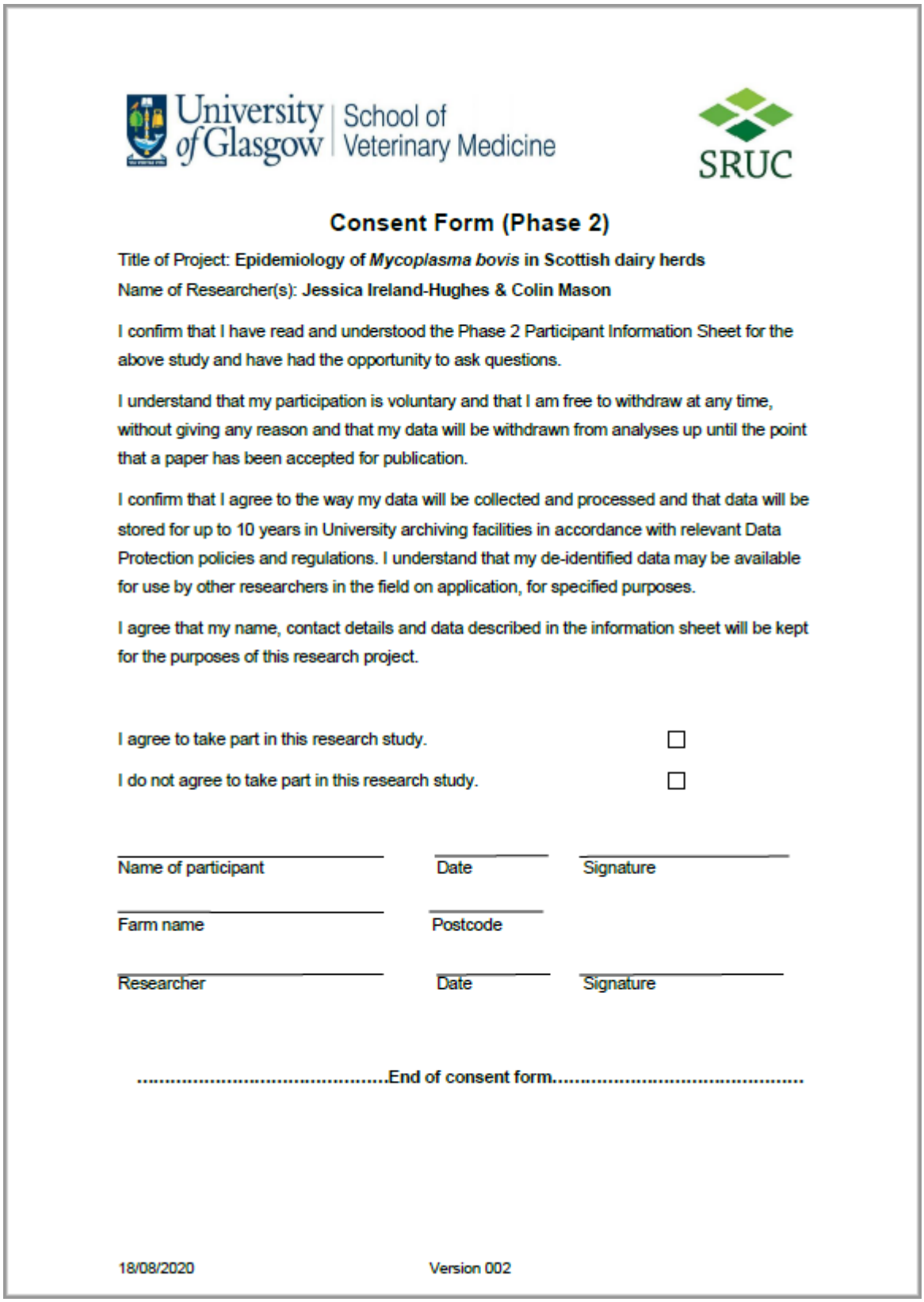
This project is funded by SRUC, in partnership with the University of Glasgow.

For further information please contact Jessica Ireland-Hughes via:
mycoplasmabovis@sruc.ac.uk or 07785 382371.

Thank you for reading this participant information sheet.

18/08/2020 Version 001

Figure A8-3 Calf study consent form



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Consent Form (Phase 2)

Title of Project: **Epidemiology of *Mycoplasma bovis* in Scottish dairy herds**

Name of Researcher(s): **Jessica Ireland-Hughes & Colin Mason**

I confirm that I have read and understood the Phase 2 Participant Information Sheet for the above study and have had the opportunity to ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and that my data will be withdrawn from analyses up until the point that a paper has been accepted for publication.

I confirm that I agree to the way my data will be collected and processed and that data will be stored for up to 10 years in University archiving facilities in accordance with relevant Data Protection policies and regulations. I understand that my de-identified data may be available for use by other researchers in the field on application, for specified purposes.

I agree that my name, contact details and data described in the information sheet will be kept for the purposes of this research project.

I agree to take part in this research study. ☐

I do not agree to take part in this research study. ☐

Name of participant _____ Date _____ Signature _____

Farm name _____ Postcode _____

Researcher _____ Date _____ Signature _____

.....End of consent form.....

18/08/2020 Version 002

Figure A8-4 Calf study sampling guidance for vets

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Mycoplasma bovis Study Sampling Guidance

Thank you for your help in researching the prevalence of *M. bovis* in Scottish dairy herds.

This is phase 2 of the study where we are exploring the link between *M. bovis* bulk milk prevalence and calf seroprevalence.

Approach

Could you please **randomly sample 20 HOMEBRED calves**:

- 10 x 4-8 months old **AND**
- 10 x 10-14 months old.

Include calves of any sex and breed (i.e., Holstein-Friesian, beef crosses, Jersey, etc.).

Be as random as you can, example scenarios:

- If you are running the calves up a race then an option could be to sample every Nth animal, e.g. every 3rd calf.
- If for example the 10-14 month old calves are split across multiple pens, please try and sample from more than one pen if you can.
- If on the day you visit and there are less than 10 calves in an age group, sample all calves in that group.

As we are testing for antibodies, if the farm uses a *Mycoplasma bovis* vaccine then they cannot participate in the study. If between registering for the study and sampling the farm introduces an *M. bovis* vaccine, please let us know.

Please can the farmer also collect a sample of the bulk milk.

Recording info

Please ensure the attached **submission form** is completed to include info on:

- Farm name & postcode
- Date of sampling
- Calf ear tag IDs written next to sample number to link the blood sample to the correct calf
- Pen number the calf was sampled from, so we know if the calves sampled came from the same pen.

Documents for farmer to complete

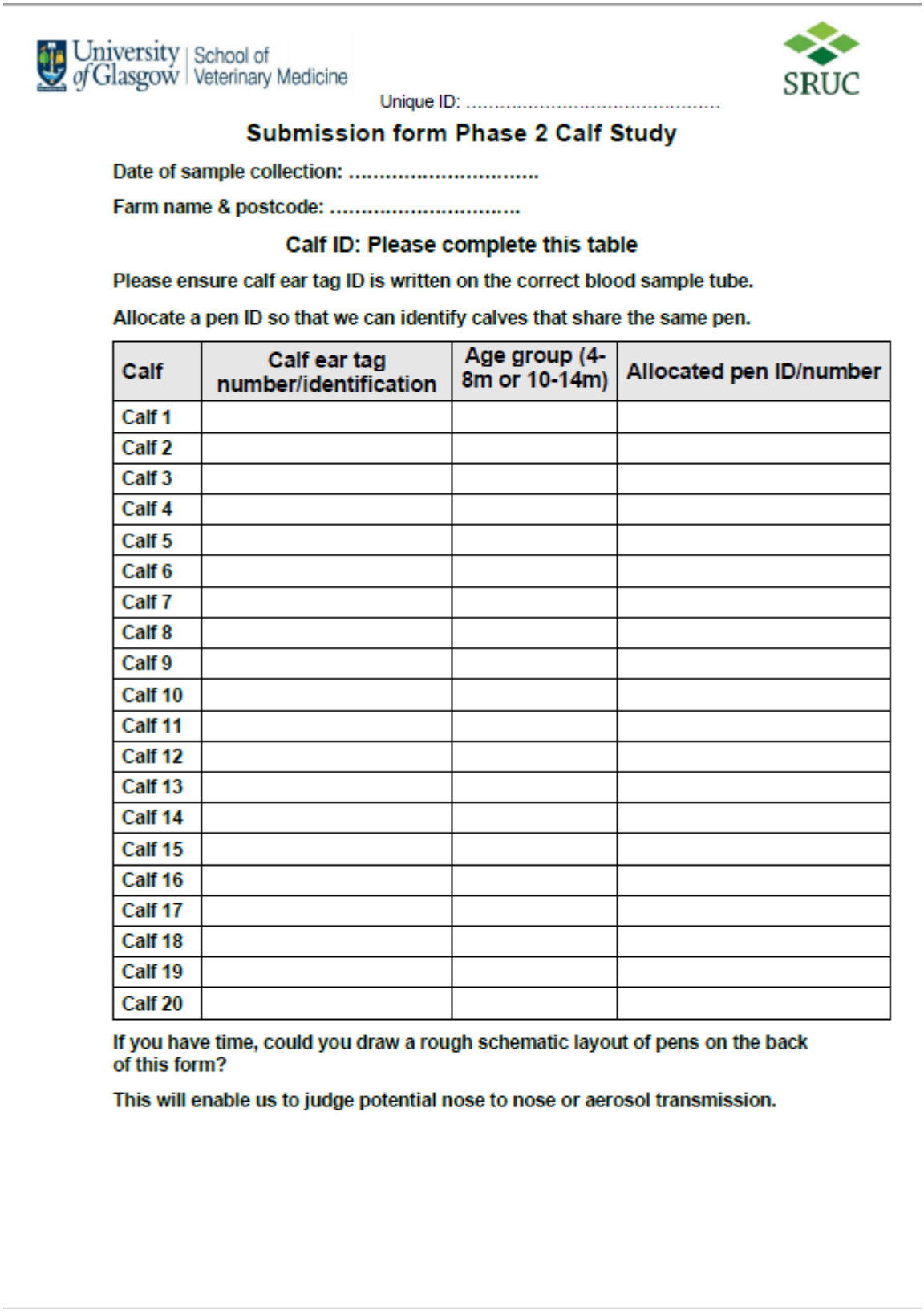
Please ensure the farmer signs and dates the **consent form** otherwise we cannot use their samples or data.

Also, if the farmer could complete the short **questionnaire**.

In summary: 10x blood samples 4-8 month old calves, 10x blood samples 10-14 month old calves, 1x bulk milk sample, consent form, sample submission form, questionnaire.

If you have any issues or questions on the day, please phone Jessica on 07785 382371 or email mycoplasmabovis@sruc.ac.uk

Figure A8-5 Calf study sample submission form



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Unique ID: .....

Submission form Phase 2 Calf Study

Date of sample collection: .....

Farm name & postcode: .....

Calf ID: Please complete this table

Please ensure calf ear tag ID is written on the correct blood sample tube.

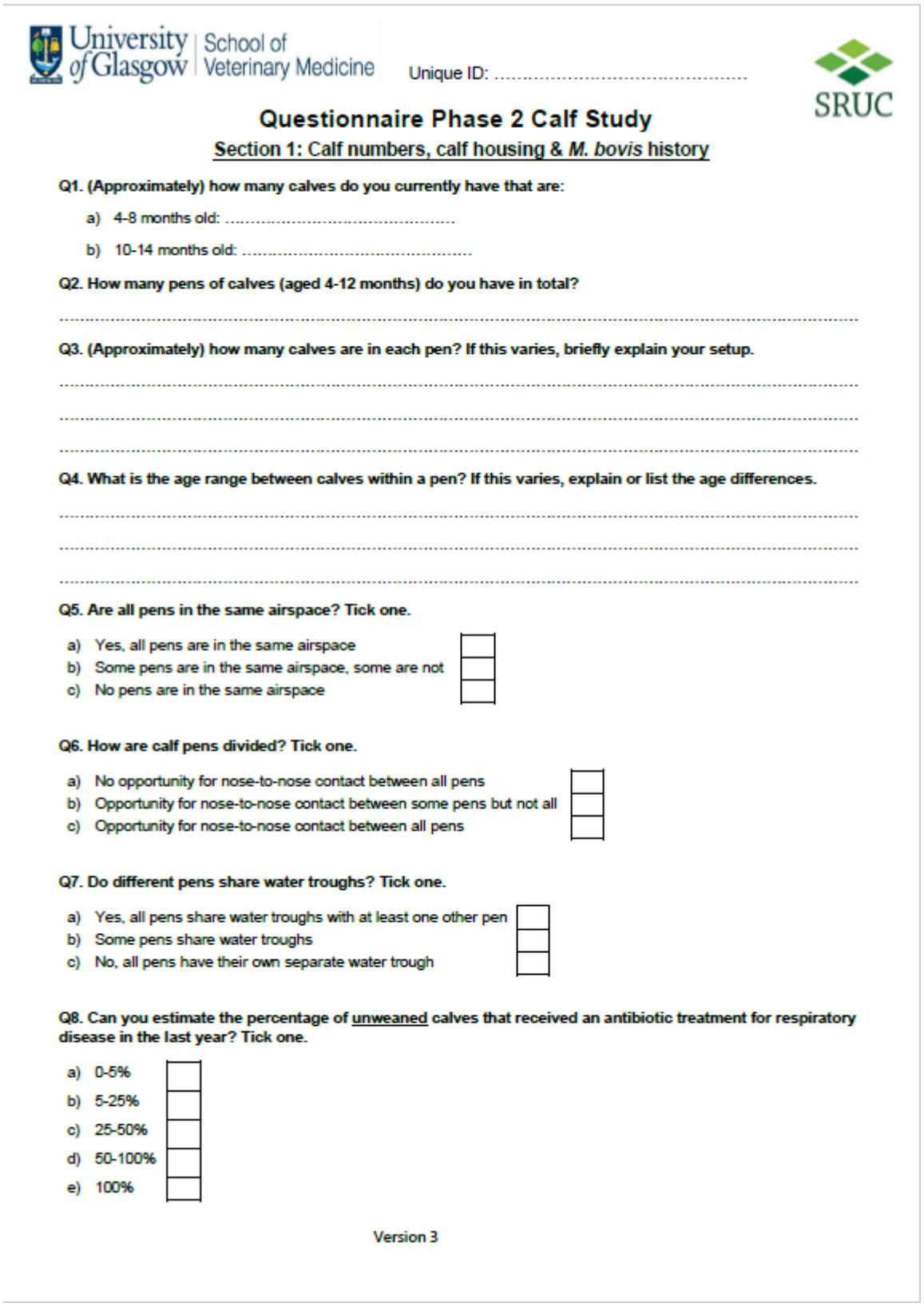
Allocate a pen ID so that we can identify calves that share the same pen.

Calf	Calf ear tag number/identification	Age group (4-8m or 10-14m)	Allocated pen ID/number
Calf 1			
Calf 2			
Calf 3			
Calf 4			
Calf 5			
Calf 6			
Calf 7			
Calf 8			
Calf 9			
Calf 10			
Calf 11			
Calf 12			
Calf 13			
Calf 14			
Calf 15			
Calf 16			
Calf 17			
Calf 18			
Calf 19			
Calf 20			

If you have time, could you draw a rough schematic layout of pens on the back of this form?

This will enable us to judge potential nose to nose or aerosol transmission.

Figure A8-6 Calf study questionnaire



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Unique ID: .....

SRUC

Questionnaire Phase 2 Calf Study

Section 1: Calf numbers, calf housing & *M. bovis* history

Q1. (Approximately) how many calves do you currently have that are:

a) 4-8 months old: .....

b) 10-14 months old: .....

Q2. How many pens of calves (aged 4-12 months) do you have in total?

.....

Q3. (Approximately) how many calves are in each pen? If this varies, briefly explain your setup.

.....

.....

.....

Q4. What is the age range between calves within a pen? If this varies, explain or list the age differences.

.....

.....

.....

Q5. Are all pens in the same airspace? Tick one.

a) Yes, all pens are in the same airspace ☐
b) Some pens are in the same airspace, some are not ☐
c) No pens are in the same airspace ☐

Q6. How are calf pens divided? Tick one.

a) No opportunity for nose-to-nose contact between all pens ☐
b) Opportunity for nose-to-nose contact between some pens but not all ☐
c) Opportunity for nose-to-nose contact between all pens ☐

Q7. Do different pens share water troughs? Tick one.

a) Yes, all pens share water troughs with at least one other pen ☐
b) Some pens share water troughs ☐
c) No, all pens have their own separate water trough ☐

Q8. Can you estimate the percentage of unweaned calves that received an antibiotic treatment for respiratory disease in the last year? Tick one.

a) 0-5% ☐
b) 5-25% ☐
c) 25-50% ☐
d) 50-100% ☐
e) 100% ☐

Version 3

Q9. Have you had *Mycoplasma bovis* diagnosed in calves in the last 12 months?

a) Yes, how were they diagnosed? Tick all that apply. ☐
   a. Post-mortem ☐
   b. Nasal swab ☐
   c. Blood sample ☐
   d. Other (describe): ☐

.....

.....

b) No ☐

Section 2: Milk and colostrum feeding

Q10a. Do you feed cows' colostrum to calves? Tick one.

a) I always feed it ☐
b) Sometimes ☐
c) Very rarely ☐
d) Never ☐

Q10b. If you feed cows' colostrum, what calves receive it, i.e. dairy heifers, dairy bulls, beef crosses, all calves?

.....

.....

Q11. What do you use as your source of cows' colostrum? Tick all that apply.

a) Mother/dam ☐
b) Another cow in the herd ☐
c) More than one cow in the herd (pooled colostrum) ☐

Q12a. Do you feed cows' milk to calves? Tick one.

a) I always feed it ☐
b) Sometimes ☐
c) Very rarely ☐
d) Never ☐

Q12b. If you feed cows' milk, what calves receive it, i.e. dairy heifers, dairy bulls, beef crosses, all calves?

.....

.....

Q12c. Do you pasteurise the cows' milk before feeding it to calves? Tick one.

a) Yes ☐
b) No ☐

Q13. At what stage is powdered milk the only source of milk fed to calves? (When do you stop feeding cows milk?) Describe:

.....

.....

Section 3: Management, breeding & calving

Q14. Do you have separate staff who milk/work with the milking herd and staff who rear the calves? Tick one.

a) We have completely separate personnel, who work with either the calves **or** the cows ☐
b) Staff usually work with calves **or** cows, but sometimes have to do both ☐
c) The same staff work with both the cows **and** the calves ☐
d) Other (describe): ☐

.....

.....

Q15. If your lactating herd are housed inside, what is the housing setup? Tick all that apply.

a) Lactating herd and calves are housed in the same shed/airspace. If so, what is the distance (approximately) between the cows and calves? (Describe below): ☐

.....

.....

.....

b) Lactating herd and calves are housed in different sheds/airspaces ☐
c) Lactating herd and calves are housed on different farms/sites ☐

Q16. What is your usual procedure for calving cows? (Tick one)

a) Move cow to an individual pen when she begins calving ☐
b) Calve in a group pen and move to individual pen after calving ☐
c) Other (describe): ☐

.....

.....

Q17. What bedding material do you use in the area that cows calve in? Tick all that apply.

a) Straw ☐
b) Sand ☐
c) Grass ☐
d) Paper ☐
e) Sawdust ☐

Q18a. What do you use for breeding? Tick one.

a) Breeding bull only ☐
b) Artificial insemination only ☐
c) Both ☐

Q18b. If you use a breeding bull,

I. **How many breeding bulls did you purchase in the last 12 months?** .....

II. **When did you purchase them?** .....

Appendix 9

Table A9-1 Number of seropositive calves within each herd by age group

Farm ID	4-8 months			10-14 months			Overall farm category
	Total	Sampled (n)	Positive (n)	Total	Sampled (n)	Positive (n)	
Mbov_0002	40	10	0	47	10	0	Neg
Mbov_0003	70	10	10	40	10	8	Pos
Mbov_0004	38	10	8	33	10	10	Pos
Mbov_0019	100	10	10	90	10	10	Pos
Mbov_0020	50	10	0	60	10	0	Neg
Mbov_0029	40	10	0	30	10	0	Neg
Mbov_0030	88	10	0	108	10	0	Neg
Mbov_0032	50	10	0	50	10	1	Pos
Mbov_0040	-	10	0	-	10	0	Neg
Mbov_0049	63	10	0	49	10	0	Neg
Mbov_0054	22	10*	0	17	10*	7	Pos
Mbov_0055	40	10	8	60	10	10	Pos
Mbov_0059	19	10	0	10	10	0	Neg
Mbov_0063	30	10	9	40	10	0	Pos
Mbov_0065	39	10	0	38	10	0	Neg
Mbov_0067	87	10	10	55	10	8	Pos
Mbov_0068	10	10	0	6	6	0	Neg
Mbov_0070	44	10	0	60	10	0	Neg
Mbov_0094	40	10	0	40	10	2	Pos
Mbov_0097	74	10	10	45	10	7	Pos
Mbov_0100	50	10	0	50	10	0	Neg
Mbov_0106	20	10	10	25	10	10	Pos
Mbov_0110	41	10	10	45	10	10	Pos
Mbov_0113	65	10	10	100	10	10	Pos
Mbov_0116	30	10	8	16	10	10	Pos

Farm ID	4-8 months			10-14 months			Overall farm category
	Total	Sampled (n)	Positive (n)	Total	Sampled (n)	Positive (n)	
Mbov_0121	55	10	8	55	10	8	Pos
Mbov_0122	28	10	10	25	10	10	Pos
Mbov_0125	28	10	0	28	10	0	Neg
Mbov_0148	121	10	0	43	10	10	Pos
Mbov_0152	35	10	0	30	10	0	Neg
Mbov_0153	15	10	10	15	10	10	Pos
Mbov_0159	48	10	0	51	10	1	Pos
Mbov_0160	45	10	0	50	10	0	Neg
Mbov_0169	16	10	10	30	10	2	Pos
Mbov_0172	30	10	10	8	10	10	Pos
Mbov_0179	10	10	0	23	10	0	Neg

*This participant did not state the age of their calves. Pos = positive, Neg = negative.

Figure A9-1 Distribution of youngstock ELISA optical density results by farm

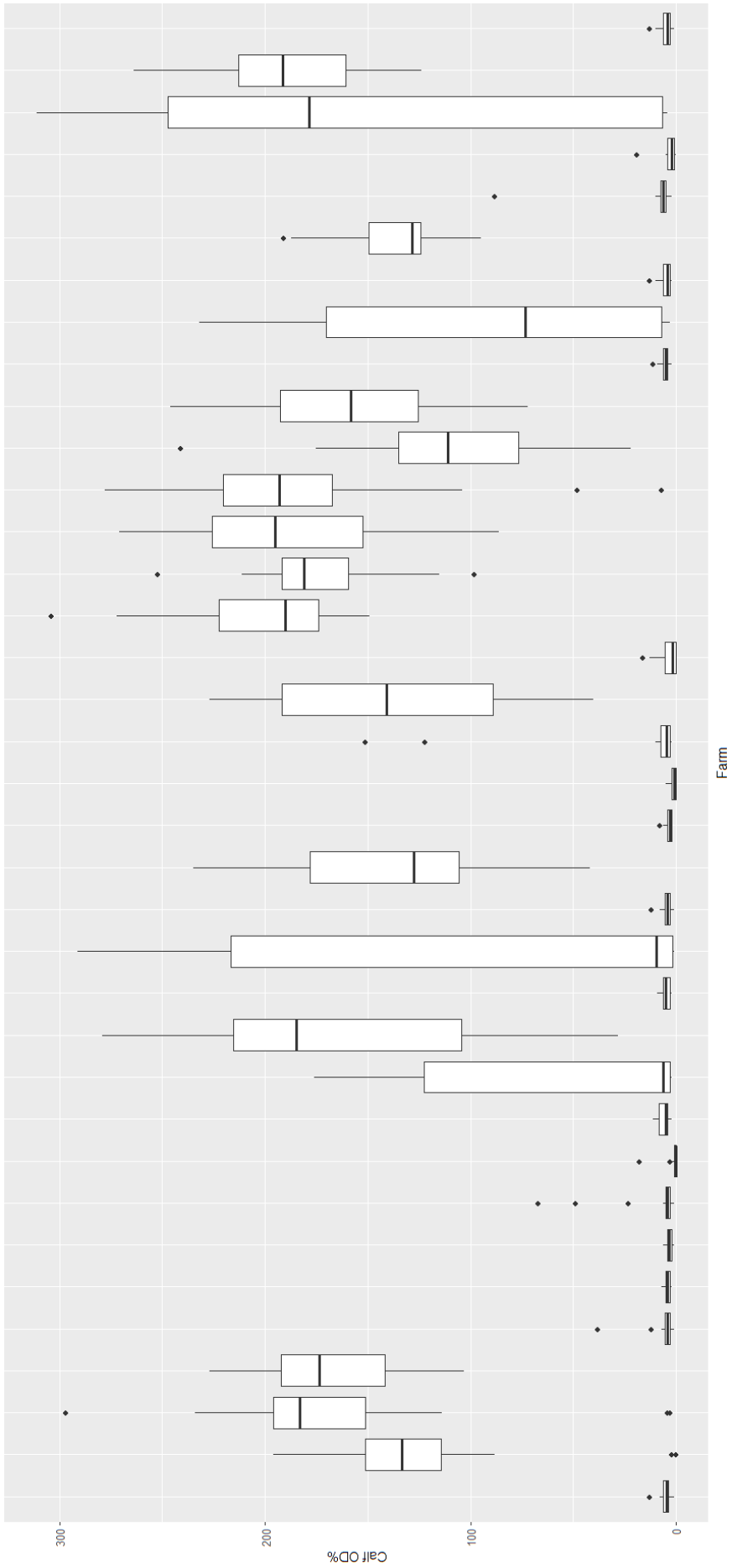
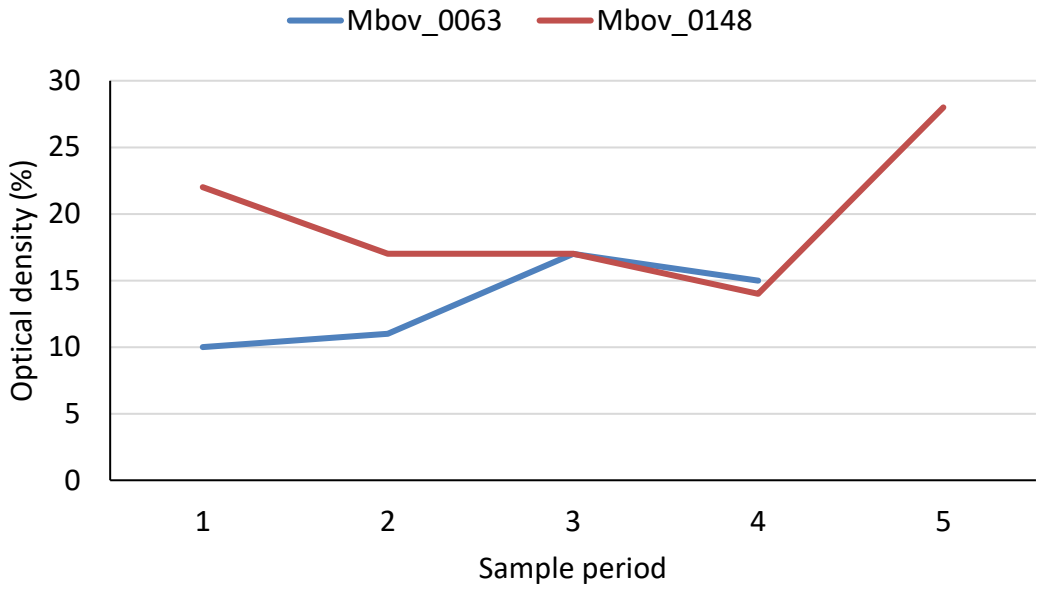


Figure A9-2 BTM optical density results of the two herds that remained negative for all samples and tested seropositive in one of the two age groups of youngstock



Appendix 10

Equation 10-1 Estimating the potential cost to dairy farms with a within-herd prevalence of *M. bovis* of 20%

- Within herd prevalence = 20% (Timonen et al., 2017)
- Reduction of daily milk yield/cow = 3.0kg (Timonen et al., 2017)
- 100-cow herd
- Average daily yield of 30l/cow
- 44 pence per litre

Average income per year of a herd with no *M. bovis*:

$$100(cows) \times 30(l/cow/day) \times 0.44(pence/litre) \times 365\ (days) = £481{,}800/year$$

Average income per year of a herd with *M. bovis* mastitis in 20% of the herd:

$$80(cows) \times 30(l/cow/day) \times 0.44(pence/litre) \times 365(days) = £385{,}400/year$$

$$20(cows) \times 27(l/cow/day) \times 0.44(pence/litre) \times 365(days) = £86{,}870/year$$

$$£385{,}400\ (80\%\ with\ no\ M.bovis) + £86{,}870\ (20\%\ with\ M.bovis) = £472{,}270/year$$

Average economic loss per year in a herd with *M. bovis* mastitis in 20% of the herd:

$$£481{,}800 - £472{,}270 = £\mathbf{9{,}530}/\boldsymbol{year\ loss}$$

Equation 10-2 Estimating the potential cost of pneumonia in youngstock

The estimated cost of pneumonia in youngstock is calculated below in a herd with 100 dairy bull beef calves. The initial parameters used to calculate the maximum profit margin in a herd with no pneumonia are shown below:.

- Herd size (number of dairy beef calves) = 100
- Age in months (days) = 6 (180)
- Average weight by 6 months of age = 220kg
- Total mixed ration (TMR) cost = £1.50/head/day
- Lightweight value = £2.15/kg

In a herd with no pneumonia, considering the cost of feed and the predicted daily liveweight gain (DLWG) the **profit margin was calculated as £15,700 per year**:

Total feed costs:

$$180 \times 100 \times 1.50 = £27{,}000$$

Predicted value of DLWG:

$$180 \times 1.0 \times 2.15 + 40 \times 100 = £42{,}700$$

Profit margin:

$$42{,}700 - 27{,}000 = £15{,}700$$

If there was pneumonia in 5% of the herd and 40% of the herd were treated for the pneumonia, the estimated losses could be between £10,000 and £11,000 per year.

Cost of a 5% mortality rate:

$$5 \times 220 \times 2.15 = £2{,}365$$

Cost of treating 40% with antibiotics:

$$40 \times 8 \times 2.92 = £934$$

Cost of treating 40% with NSAID:

$$40 \times 8 \times 0.74 = £237$$

Total cost of treating 40%:

$$934 + 237 = £1{,}171$$

Cost of an average reduced growth rate of 0.2kg/day:

$$0.2 \times 180 \times 2.15 \times 95 = £7{,}353$$

Total estimated losses due to undifferentiated pneumonia:

$$2{,}365\ (mortality) + 1{,}171\ (treatment) + 7{,}353\ (growth) = £10{,}889$$

Estimated profit of £4,811 in a herd with pneumonia.

References

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