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Mycoplasma bovis in Swedish dairy herds

Prevalence, epidemiology and prospects for control

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Mycoplasma bovis in Swedish dairy herds,
Prevalence, epidemiology and prospects for control

Abstract

Mycoplasma (M.) bovis is an important pathogen causing mastitis, pneumonia, arthritis and otitis media in cattle throughout the world. The aim of this thesis was to gain further knowledge of the prevalence and epidemiology of this infection in Swedish dairy herds, and to evaluate testing strategy to detect infected herds.

The first study was a national screening investigating prevalence of *M. bovis* antibodies and bacterial DNA in BTM in all Swedish dairy herds. We found large regional variations with an apparent prevalence of 3-20% in the southern regions. Large herd size was a risk factor for being antibody positive in BTM and positive herds had a higher calf- and youngstock mortality (2-15 months of age).

In the second study, we examined the dynamics of *M. bovis* over time in herds (n=149) in the south of Sweden. Finding seropositive herds increased when adding samples from primiparous (PP) cows. Large herd size and introduction of cattle were risk factors associated with higher levels of *M. bovis* antibodies in PP cows.

In the third study, a questionnaire was used to investigate association between herd-level antibody status and biosecurity- and management routines. In the participating herds (n=115), being affiliated to the biosecurity program "Smittsäkrad besättning" was associated with *M. bovis* antibody negative status.

The fourth study investigated the patterns of antibody prevalence over time in cows and calves in 35 herds. The association of antibody status in individual cows and milk production was quantified, and we found a reduction in milk production in antibody positive cows. The best testing strategy to find infected herds was to analyse antibodies both in BTM samples and in individual cow samples.

The results of this thesis indicate that cost-effective strategies for controlling *M. bovis* infections could be developed, based on biosecurity and antibody monitoring. Implementing such a control strategy would improve cattle health in Sweden.

Keywords: *M. bovis, Mycoplasmopsis bovis*, antibody detection, regional variation, risk factors, milk production, biosecurity, cattle, control, herd health

Mycoplasma bovis i svenska mjölkbesättningar

Förekomst, epidemiologi och möjligheter till kontroll

Sammanfattning

Mycoplasma (M.) bovis är en viktig patogen som orsakar mastit, lunginflammation, artrit och otitis media hos nötkreatur världen över. Syftet med denna avhandling var att få ytterligare kunskap om prevalens och epidemiologi i svenska mjölkbesättningar och utvärdera provtagningsstrategi för att identifiera smittade besättningar. Den första studien var en nationell undersökning av förekomst av *M. bovis*-antikroppar och bakteriellt DNA i tankmjölk (BTM) hos alla svenska mjölkbesättningar. Vi fann stora regionala variationer, med en prevalens på 3–20% i de södra regionerna. Större besättningsstorlek var en riskfaktor för att vara antikroppspositiv och positiva besättningar hade högre kalv- och ungdjursdödlighet (2–15 månader). I den andra studien undersöktes dynamiken hos *M. bovis*-antikroppar över tid i besättningar (n=149) i södra Sverige. Förekomsten av infekterade besättningar ökade när prover från förstakalvare (PP) inkluderades. Större besättningsstorlek och införsel av nötkreatur var riskfaktorer som var associerade med högre nivåer av *M. bovis*-antikroppar i PP kor. I den tredje studien användes en enkät för att undersöka sambandet mellan antikroppsstatus och besättningens skötsel- och smittskyddsrutiner. Gårdarnas anslutning till programmet Smittsäkrad besättning visade ett samband med negativ antikroppsstatus. Den fjärde studien undersökte antikroppsnivåer över tid hos kor och kalvar i 35 besättningar. Sambandet mellan antikroppstatus hos enskilda kor och mjölkproduktion kvantifierades och visade en minskning i mjölkproduktionen för antikroppspositiva kor. Den bästa provtagningsstrategin för att hitta infekterade gårdar var att analysera antikroppar i både BTM och i mjölk från enskilda kor. Resultaten av denna avhandling indikerar att kostnadseffektiva strategier för att kontrollera *M. bovis*-infektioner kan utvecklas, baserat på smittskydd och provtagning. Genomförandet av en sådan kontrollstrategi skulle förbättra hälsan hos nötkreatur i Sverige.

Keywords: *M. bovis, Mycoplasmopsis bovis*, antikroppar, regional variation, riskfaktorer, mjölkproduktion, biosäkerhet, nötkreatur, kontroll, besättningshälsa

Preface

The work presented in this thesis was conducted from 2019 to 2025, a period characterised by the Covid-19 pandemic, and in cattle the spread of Bluetongue virus type 3 in Europe. In Sweden, *Mycoplasma (M.) bovis* can cause serious disease especially in calves in fattening herds. This was not a new disease problem when this research work commenced, but there were indications of increasing *M. bovis* infections in the country. The epidemiological knowledge was brief and predominantly built on field reports. This thesis focused on the potential spread of *M. bovis* infection in Swedish dairy herds, but all cattle can be infected.

This research is not the only work that has been performed in Sweden to enhance the knowledge about this disease. The organisations Växa Sverige, Farm and Animal Health, Skånesemin and Distriktsveterinärerna have carried out several research projects, spread information to both farmers and veterinarians, and worked together for an increased awareness of this disease in Sweden. This has also included advice about prevention and how to manage *M. bovis* positive herds, for dairy, beef, and suckler herds.

Collaboration between industry and university is essential to control infectious diseases. This has been a significant strength in this thesis work, and it has been possible to implement the results early in cattle herds.

Dedication

To my family

To the cows

”Och allt är så enkelt,
så enkelt och stort,
Det finns i ett koöga, alltid.”
Lars Lundkvist, Koöga (dikter, Norstedts, 1977)

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Hurri E, Ohlson A, Lundberg Å, Aspán A, Pedersen K, Tråvén M. (2022). Herd-level prevalence of *Mycoplasma bovis* in Swedish dairy herds determined by antibody ELISA and PCR on bulk tank milk and herd characteristics associated with seropositivity. Journal of Dairy Science, vol 105 (9), p7764-7772. https://doi.org/10.3168/jds.2021-21390

II. Hurri E, Alvåsen K, Widgren S, Ohlson A, Aspán A, Pedersen K, Tråvén M (2025). A longitudinal study of the dynamics of *Mycoplasma bovis* antibody status in primiparous cows and bulk tank milk in Swedish dairy herds. Journal of Dairy Science, vol 108 (1), p845-855. https://doi.org/10.3168/jds.2024-25304

III. Alvåsen K., Hurri E., Magnusson H., Tråvén M. Management and biosecurity practices associated with *Mycoplasma bovis* seropositivity in Swedish dairy herds: a questionnaire study. (manuscript)

IV. Hurri E., Compton C.W.R., Alvåsen K. Tråvén M. Patterns of *Mycoplasma bovis* antibodies in cows and calves in Swedish dairy herds and testing strategies to detect seropositive herds. (manuscript)

All published papers are published open access.

The contributions of Emma Hurri (EH) to the papers included in this thesis were as follows:

I. EH contributed to the design and performance of the statistical analyses and drafted and finalised the manuscript.

II. EH contributed to the design of the study, compiled and cleaned the data, performed the statistical analyses, and drafted and finalised the manuscript.

III. EH contributed to the design of the study and to the analyses, organised the construction and administration of the questionnaire, and partly drafted and finalised the manuscript.

IV. EH contributed to the design of the study, compiled most of the data, performed the statistical analyses with support from an epidemiologist, and drafted and finalised the manuscript.

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Abbreviations

BTM	Bulk tank milk
CDB	Central register of bovine animals
DHI	Dairy Herd Improvement
DNA	Deoxyribonucleic Acid
MP	Multiparous
OD	Optical density
PCR	Polymerase Chain Reaction
PP	Primiparous
S/P	Sample to positive
SCC	Somatic cell count
SLU	Swedish University of Agricultural Sciences
SVA	Swedish Veterinary Agency

1. Introduction

Mycoplasma (M.) bovis is an emerging pathogen responsible for severe diseases in cattle worldwide (Maunsell et al., 2011), including pneumonia, mastitis, arthritis, and middle ear infections (Nicholas and Ayling, 2003). Over the past decade, *M. bovis* has spread to new regions, initially detected in Sweden in 2011 (Ericsson Unnerstad et al., 2012), followed by Finland in 2012 and New Zealand in 2017 (Vähänikkilä et al., 2019, Dudek et al., 2020). This bacterium is naturally resistant to penicillin and infections often fail to respond to broad-spectrum antibiotics, resulting in chronic disease that compromises animal welfare and causes substantial economic loss for the cattle industry (Nicholas and Ayling, 2003).

A national screening conducted in Sweden in 2016, using PCR analysis on bulk tank milk (BTM), revealed an apparent prevalence of 0.3% (n = 10) for *M. bovis* (Landin et al., 2019). There were also field reports on pneumonia in calves and a few mastitis cases. However, aside from this, knowledge about *M. bovis* in Swedish cattle herds was very sparse. When this thesis work commenced in 2019, *M. bovis* was regarded as a relatively new and rare disease. There was a significant need to raise awareness and enhance knowledge about its prevalence and how to prevent disease spread. To develop effective control strategies for *M. bovis*, the epidemiology must be investigated, including the identification of risk factors for transmission and estimations of the impact on herd health and performance.

The overall aim of this doctoral research was to gain knowledge about the epidemiology of *M. bovis* infections in Swedish dairy herds. Chapter 2 provides a general background of the bacterium *M. bovis* and *M. bovis* infections in cattle. Chapters 3–6 are devoted to the studies conducted during this doctoral work and chapter 7 summarises the areas that were identified for further research.

2. Background

2.1 Beef and Dairy cattle in Sweden

In 2020, at the start of this thesis work, there were approximately 1 450 000 cattle in Sweden. Of these, 300 000 were dairy cows and 210 000 were suckler cows. The dairy cows were to be found in 3090 herds with an average herd size of 98 cows. The number of both dairy herds and cows have been decreasing for several decades; 20 years ago, in 2000, there were about 430 000 dairy cows in 12 680 herds, with an average herd size of 34 cows (Swedish Board of Agriculture, 2020). The number of cows within Sweden is continuing to decrease and the most recent statistics from June 2024 revealed that there were approximately 1 410 000 cattle, 290 000 dairy cows and 200 000 suckler cows. However, the average dairy herd size has increased to 113 cows (Swedish Board of Agriculture, 2024). Amongst dairy herds, Swedish Holstein (SH) and Swedish Red (SR) are the two most common breeds (80%), and their average milk yield in kg ECM per cow and year was 10 790 for SH and 9 910 for SR in 2020. The Dairy Herd Improvement (DHI) database collects and processes information about production, fertility, and health in dairy herds, and 77% of the cows in Sweden were affiliated to the DHI in 2020 (Cattle Statistics, Växa, 2020). Sweden has a favourable situation regarding animal health as many infectious diseases have been eradicated, for example bovine viral diarrhea virus (BVDV), enzootic bovine leukosis (EBL), infectious bovine rhinotracheitis (IBR), and bovine tuberculosis, brucellosis, and paratuberculosis. This has been achieved through efficient control and surveillance programmes managed in joint effort with industry and authorities (Cattle Health Statistics, Växa, 2019-2020). Swedish dairy cows

are healthy, the disease incidence has continually been decreasing and was 20 cases per 100 cows per year in 2020. For dairy calves, the calf mortality for calves born alive was 2.5% during the first month of life in 2020 (Animal Health, Swedish Board of Agriculture 2020). Around two thirds of bull calves born in a dairy herd are sold to a herd specialised in meat production, and one fifth of those calves are sold in their first month in life, with the rest being sold after weaning at 2-3 months of age (Structure of the Swedish cattle sector, Swedish Board of Agriculture, 2022). Sweden's agricultural industry is primarily concentrated in the southern and central regions of the country, with the largest sectors being meat (all livestock) and dairy production, Figure 1 provides an overview of the cattle population in Sweden 2020 (National Veterinary Institute, 2020).

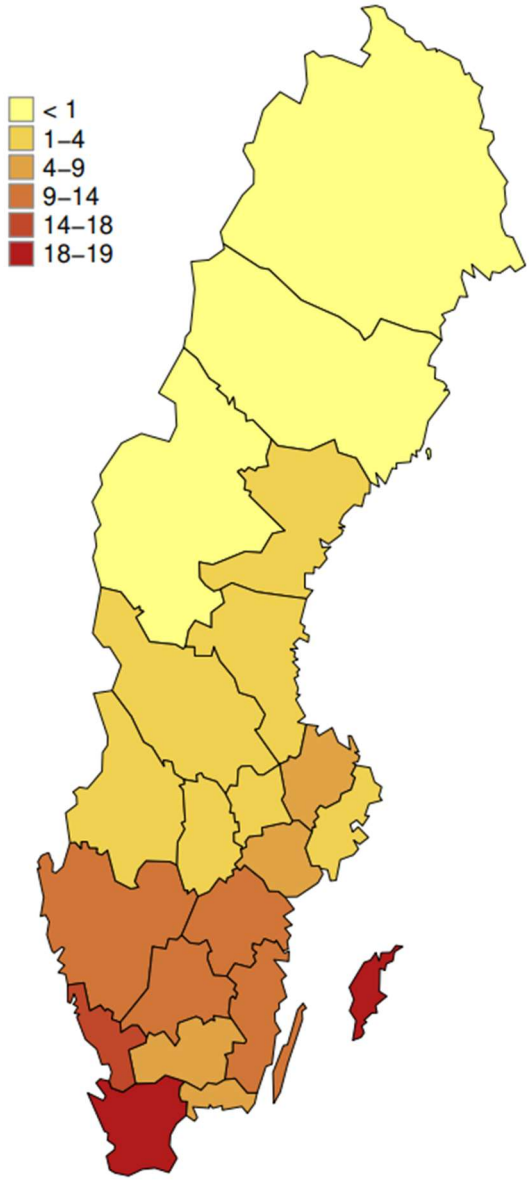

Figure 1. Number of cattle per km^2 in 21 Swedish regions as of June 2020 (Surveillance of infectious diseases in animals and humans in Sweden 2020, National Veterinary Institute (SVA), Uppsala, Sweden. SVA:s rapportserie 68 1654-7098).

2.2 *Mycoplasma bovis*

2.2.1 Aetiology

Mycoplasmas belong to the class *Mollicutes* (soft skin in Latin), they lack a cell wall and are the smallest self-replicating living organisms (Razin et al., 1998). The genus *Mycoplasma* includes over 100 species, 13 of which are recognised as cattle pathogens (Nicholas et al., 2008). The taxonomy of mycoplasmas is controversial with vaying opinions among research groups. A revision of the taxonomy based on whole genome sequencing has been suggested along with a new name, *Mycoplasmopsis bovis* (Gupta et al., 2018). However, a recently published paper advised keeping the former taxonomy with the current name (Yan et al., 2024). The two most important cattle pathogens in this group of bacteria are *M. bovis* and *M. mycoides* subsp *mycoides*, both of which cause serious and economically costly diseases in cattle (Nicholas and Ayling, 2003). *Mycoplasma bovis* is specifically adapted to cattle, although it has occasionally been isolated in chickens, buffaloes, small ruminants, and even humans (Pitcher and Nicholas, 2005). The *M. bovis* bacteria have an affinity for mucous membranes where they can exist as commensals or pathogens. The predilection site is the upper respiratory tract, urogenital tract, and mammary gland (Maunsell et al., 2011). In cattle naturally infected with respiratory disease, *M. bovis* is frequently found in conjunction with other microorganisms, leading to the hypothesis that synergism may contribute to the severe lung lesions that can be observed. The most common bacterial and viral pathogens seen in association with *M. bovis* are *Pasteurella multocida*, *Mannheimia haemolytica*, bovine respiratory syncytial virus (BRSV), bovine herpes virus 1 (BHV-1) and parainfluenza virus type 3 (Bürki et al., 2015, Caswell et al., 2010). The most common respiratory viruses amongst cattle in Sweden are BRSV and bovine corona virus, with 75-100% of dairy herds being antibody positive yearly (Ohlson et al., 2013). A recent experimental study showed that coinfection with influenza D virus and *M. bovis* increased the severity of the respiratory disease in calves compared to infection with one of the pathogens, influenza D or *M. bovis* (Lion et al., 2021).

2.2.2 History

Mycoplasma bovis was first isolated from a mastitis outbreak in the USA in 1961 (Hale et al., 1962). The bacteria then appear to have spread via animal

movements to many countries and was detected in; Israel (1964), Spain (1967), Great Britain (1975), Germany (1977), Denmark (1981) and Brazil (1989) (Nicholas and Ayling, 2003, Dudek et al., 2020, Friis and Krogh, 1983). This spread occurred through trading with genetically valuable cattle. Finland and New Zealand are two countries that experienced a more recent introduction and reported their first *M. bovis* diagnosis in 2012 and 2017, respectively (Haapala et al., 2018, Jordan et al., 2021).

In Sweden there were a few historic cases in the spring and summer of 1988. *Mycoplasma bovis* was detected in pneumonia cases in calves in two herds in the western part of Sweden (Västergötland) (Bölske, 1988). This pathogen had not been previously detected in Sweden. The possible risk of spreading the infection through semen resulted in an investigation of the infection status at the semen production centres by the Swedish Board of Agriculture and Swedish Veterinary Agency (SVA). All bulls, including recruitment bulls, were sampled with swabs from both nasal cavities, and raw semen if existing. The samples were placed in mycoplasma transport substrate and cultured at the Mycoplasma laboratory at SVA. The bacteria were found in the upper respiratory tract in 21% (n=20) of the bulls at the individual assessment stable at one of the semen production centres. All semen samples were negative. The positive bulls were re-tested with nasal swabs before entering the semen production, and they all became negative eventually, although this took several months. There had been severe respiratory disease at this semen centre in the winter of 1987/1988 and 1988/1989, six young bulls (3-5 months) had been autopsied and were positive for *M. bovis* at culture. In addition, eight calves with pneumonia had been *M. bovis* positive in lung samples from autopsy, seven from Västergötland and one from Hudiksvall (unpublished G. Bölske, 1990). The last *M. bovis* positive case historically was a calf with pneumonia in 1996.

In 2011, *M. bovis* was diagnosed again in Sweden and this time also from cows with mastitis, besides calves with respiratory disease (Ericsson Unnerstad et al., 2012). In a study investigating the genetic relatedness between *M. bovis* isolates from the Nordic countries and other European countries, a new clone dominated in the Nordic countries in the isolates collected from 2011 and onwards (Tardy et al., 2020).

Undoubtedly, *M. bovis* is considered as a significant pathogen in cattle, responsible for diseases that result in considerable harm to animal welfare

and substantial economic losses in farming (Calcutt et al., 2018, Nicholas, 2011).

2.2.3 Pathogenesis

Mycoplasma bovis can adhere to epithelial cells, a crucial step for its colonisation and subsequent infection (Radostits, 1999). To evade the immune system, *M. bovis* has several strategies, which includes an ability to penetrate and survive in various cells, besides epithelial cells, also in peripheral blood mononuclear cells, alveolar macrophages and erythrocytes (Perez-Casal, 2020). The bacteria possess a complex system of surface lipoproteins, which are utilized in distinctive strategies to generate and maintain surface diversity, thereby offering a protective shield against the host immune system (Rosengarten et al., 2000). The host immune response is often ineffective in clearing *M. bovis* infections and may, in fact, even contribute to the pathogenesis of the disease (Maunsell and Chase, 2019). The formation of biofilms is another effective way of avoiding the immune response for *M. bovis* and also suggests a capability to survive in the environment and contribute to persistence in the host (McAuliffe et al., 2006). These traits of immune evasion and immune modulation help the bacterium to persist in the host and cause chronic infections.

The incubation period for infection is challenging to define, as it varies depending on the age of the animal and the clinical manifestations of the infection. In experimental infections, the incubation period for mastitis was a few days, whilst for pneumonia, it was approximately 7 days (Calcutt et al., 2018). In a herd with an outbreak of mycoplasma mastitis, the incubation period was estimated to be 13.6 days (Punyapornwithaya et al., 2011). Shedding of *M. bovis* can be intermittent which also makes diagnosis by detection in individual animal variable in success (Biddle et al., 2003). Thus, herd diagnosis, especially when using more cost-effective serological methods, may be more reliable for detecting persistent infections. Cattle can also shed the organism for months, or possibly even years, which can be a source of reinfection in the herd and may lead to outbreaks of clinical disease (Biddle et al., 2003, Bayoumi et al., 1988).

2.2.4 Transmission

A new infection of *M. bovis* in a previously free herd usually occurs through the introduction of asymptomatic carrier animals. Transmission can be delayed until shedding occurs, if at all and this delay can complicate the identification of the infection source, leading to mycoplasma disease outbreaks in herds that appear to be closed (Maunsell et al., 2011). In New Zealand, most cases identified at the time of study were associated with animal movements, indicating that this is the predominant mode of transmission between herds (Jordan et al., 2021). Infected semen used in artificial insemination has been linked to introduction of *M. bovis* in two herds in Finland (Haapala et al., 2018).

Within-herd, *M. bovis* is transmitted from infected to uninfected cattle via nose-to-nose contact, droplets, or udder-to-udder transmission during milking (Maunsell et al., 2011, González and Wilson, 2003). Calves can become infected via ingestion of contaminated milk, although colostrum seems to contain a low number of bacteria (Maunsell et al., 2012, Gille et al., 2020). In a study investigating clinical outbreaks of *M. bovis* among adult dairy cows, the disease also spread to calves and youngstock. Transmission pathways were linked to both internal and external biosecurity measures, as well as indirect transmission routes such as feed and water stations (Biesheuvel et al., 2024, Penterman et al., 2022). *Mycoplasma bovis* can survive in the environment especially in cool and humid conditions, and survival in sand bedding was reported to be several months (Justice-Allen et al., 2010). Indirect transmission from the environment could be a source of disease, as veal calves were likely infected from contaminated cages and mangers in an Italian herd (Piccinini et al., 2015). This highlights the importance of breaking the infection route with an all-in, all-out system and effective cleaning and disinfection between animal groups. In the New Zealand *M. bovis* eradication programme, cleaning and disinfection of the barn together with a 60-day stand-down is mandatory after depopulation of infective cattle and before repopulation (Ministry for Primary Industries, 2025).

2.2.5 Clinical picture

The clinical disease caused by *M. bovis* can vary widely and includes mastitis, pneumonia, arthritis, genital disorders, and keratoconjunctivitis (Nicholas and Ayling, 2003). Additionally, otitis media can be seen in

younger animals (Maunsell et al., 2012). Mastitis caused by *M. bovis* can be subclinical, clinical, or chronic, and represents a significant challenge for both production and animal welfare in dairy herds in the US. In Europe and the Middle East, *M. bovis* mastitis is also a serious disease, but it is typically more sporadic (Calcutt et al., 2018). Mastitis caused by *M. bovis* is contagious, often more than one quarter of a herd is affected, there is loss in milk production, failure of antibiotic treatment, and infected cows can remain asymptomatic with few clinical signs (Nicholas et al., 2016). There is also an increase in somatic cell count (SCC) due to *M. bovis* mastitis (Gelgie, 2024).

Clinical symptoms of *M. bovis* pneumonia are non-specific and include fever, coughing, dyspnoea, anorexia and depression. Signs that suggest a suspicion of *M. bovis* are arthritis, otitis and failure to respond to antibiotic treatment (Caswell and Archambault, 2007). Bovine respiratory disease (BRD) is a multifactorial disease that not only involve pathogens such as *M. bovis*, but also management and environmental factors (Calcutt et al., 2018). For calf pneumonia, the mortality can be 5-10% or higher, and morbidity can be up to 35% (Nicholas et al., 2008). Older cattle can also be infected with *M. bovis* in the respiratory tract, as *M. bovis* was frequently detected in the lung tissue of cows with fatal pulmonary disease in a Brazilian dairy herd (Oliveira et al., 2020). However, calves can remain healthy even when infected with *M. bovis*, i.e. positive samples from the nasal cavity or bronchoalveolar lavage, suggesting that *M. bovis* may not cause pneumonia in all infected calves (Caswell and Archambault, 2007).

Arthritis associated with *M. bovis* is typically sporadic, though outbreaks can still occur (Maunsell et al., 2011). Affected animals become severely lame, showing signs of swollen joints and tendon sheaths, and they often respond poorly to antibiotic treatment, leading to the culling of the animals (Gagea et al., 2006, Wilson et al., 2007). As a result, arthritis represents both an animal welfare concern and a financial burden for farms. Several years ago, arthritis was the most common *M. bovis*-related disease in Danish farms, where a recent outbreak had occurred (Jensen, 2015).

2.2.6 Immune response

The innate immune response is critical as a first defence against *M. bovis*. Alveolar macrophages play a crucial role in the early clearance of mycoplasmas from the lungs (Maunsell et al., 2011). As described earlier

(pathogenesis) *M. bovis* has several mechanisms to avoid and modulate the host's immune system. Neutrophils are important in the innate immune response and are recruited to sites of inflammation in lungs and mammary gland, although this can also lead to an overload of neutrophils and more severe mycoplasma disease (Gagea et al., 2006, Rodríguez et al., 1996, Kauf et al., 2007).

There is generally also a strong humoral immune response exerted from *M. bovis* respiratory infection and mastitis. Experimental infection with *M. bovis* in gnotobiotic calves induced an IgM antibody response within 7 days, followed by an IgG response in serum two weeks later. There was also an IgA response in the upper and lower respiratory tract (Howard et al., 1986). In another experimental infection in 5- to 6-month-old calves, IgG antibodies increased 7 days post-immunization with a novel live vaccine and continued to increase for two to three weeks (Zhang et al., 2014). In calves, experimental lung infection with *M. bovis* results in a Th2-skewed immune response, which is less effective in pathogen clearance (Vanden Bush and Rosenbusch, 2003). Natural infection seems to have a more variable intensity of antibody responses than experimental infection. Young calves, less than 3 months old, had difficulty producing a detectable systemic antibody response (Virtala et al., 1996). Maternal antibodies can influence the results of serological tests and the success of vaccination in young calves. There are a few studies on maternal antibodies, for instance, in France 2.2% of veal calves were reported to have maternal antibodies (Arcangioli et al., 2008). However, these antibodies in young calves offer minimal protection against BRD, as seropositivity to *M. bovis* made no difference in the severity of respiratory disease (Pardon et al., 2015). In experimental intramammary infection both systemic and milk IgG1 and IgG2 responses and local IgA in the mammary gland are stimulated (Bennett and Jasper, 1980, Boothby et al., 1987). However, more knowledge is needed to identify the immune responses that effectively clear *M. bovis* from the respiratory tract and mammary gland, as well as the responses that provide protection from new infections (Maunsell and Chase, 2019).

The longevity of antibodies against *M. bovis* is not well understood, several studies indicate that the antibody levels can remain detectable for extended periods. In a Finnish study, antibodies were detectable for at least 1.5 years, regardless of whether *M. bovis* bacteria were found on the farm (Vähänikkilä et al., 2019). Replacement heifers exposed to M. bovis as

calves had varying persistence of antibodies and some maintained detectable levels for 2 years (Hazelton et al., 2020a). Antibodies against *M. bovis* were detected in calves from 3 weeks of age using the MilA ELISA and stayed above the cut-off during the 3-month long study (Petersen et al., 2018). In an outbreak study, herds were followed for three months and at the end, the clinical cases had vanished, yet all of the herds were positive for *M. bovis* antibodies (Penterman et al., 2022). *M. bovis* circulating within the herd could also lead to re-infection and a boost in antibody levels, therefore antibody duration is difficult to establish in naturally infected animals. However, serology offers a valuable tool for evaluating recent exposure to *M. bovis* in herds or specific groups of animals within herds (Parker et al., 2018).

2.2.7 Diagnostic methods

Diagnosing *M. bovis* infection requires laboratory confirmation as the clinical signs are not pathognomonic. Detection of the bacteria or bacterial nucleic acid can be carried out by microbial culture, or PCR analysis respectively. Antibody testing can also be used in *M. bovis* diagnostics and including clinical signs and herd history has been suggested as the most appropriate way to characterize disease status in a cattle population (Parker et al., 2018).

Culture of *M. bovis* is laborious and time consuming (7 - 10 days) and requires a post-culture identification test to differentiate it from other mycoplasmas such as *M. dispar* or *M. bovirhinis*. The advantage of culture is that it can provide clinical isolates for further investigation through whole genome sequencing (WGS) and testing of antimicrobial resistance (Calcutt et al., 2018).

PCR-based techniques have contributed significantly to an improved diagnosis of *M. bovis*, since they allow for rapid (i.e. within a few hours), sensitive and specific detection of *M. bovis* (Parker et al., 2018). A qPCR method specific for *M. bovis* that can detect bacterial loads in milk as low as 100 cfu/mL of milk has been documented (Sachse et al., 2010). An evaluation of PCR methods used in six different European laboratories revealed a large variation of both DNA extraction methods and modified in-house PCR methods (Wisselink et al., 2019). However, despite the variation they all had comparable diagnostic performances.

Measuring antibodies by enzyme-linked immunosorbent assay (ELISA) has several advantages: the animal does not need to be shedding the bacteria at the time of sampling, blood or milk samples are easy to collect, and it can measure past exposure. An indirect IgG ELISA using a membrane protein with lipase activity, mycoplasma immunogenic lipase A (MilA) was developed in Australia and showed high sensitivity (92.7%) and high specificity (98.7%) (Wawegama et al., 2014). In a field evaluation, in feedlot cattle, of the MilA ELISA, the diagnostic sensitivity was 94.3% and specificity was 94.4% (Wawegama et al., 2016). There is since 2019 a commercial indirect IgG ELISA, IDscreen® Mycoplasma bovis (IDvet, Grabels, France) available, which is based on the MilA method developed by Wawegama et al. (2014). Several recent studies have evaluated the IDvet ELISA using Bayesian latent class analysis, and the IDvet ELISA performed with higher precision and accuracy compared to Bio-X ELISAs (BIO K302, BIO K260) and Western blot, sensitivity (92.5%-93.5%) and specificity (98.6%-99.3%) (Veldhuis et al., 2023, Andersson et al., 2019). A field study comparing the performance of IDvet ELISA on serum and milk samples showed a high correlation, which implies that milk samples can replace serum samples for *M. bovis* diagnostics in adult cows (Petersen et al., 2020). Using BTM samples for herd-level diagnosis is a cost-effective tool when screening for *M. bovis* positive herds. In evaluations of antibody ELISA tests for *M. bovis* antibodies in BTM using Bayesian latent class analysis, the IDvet/MilA ELISA had best performance with high sensitivity (94%-96.6%) and high specificity (92%-94.2%) (Salgadu et al., 2022, McAloon et al., 2024).

2.2.8 Prevalence

M. bovis is endemic in many of the cattle-producing countries in the world. Finland, Sweden, and New Zealand are exceptions with a relatively low prevalence, and Norway remain free of *M. bovis*.

Prevalence of *M. bovis* varies significantly among countries and between herds. In Israel the herd-level prevalence of *M. bovis* mastitis was low between 2004-2007 (0-0.68%), increased in 2008 to 3.77% due to a mastitis outbreak, and since 2008 new *M. bovis* positive herds have been identified annually using PCR-analysis on BTM and mastitis samples (Lysnyansky et al., 2016). In the Netherlands, the prevalence was 7% PCR positive and 17% Ab ELISA-positive, using BIO K302 ELISA, on randomly selected herds

tested on BTM in 2016 (Gille et al., 2018). In Denmark, the apparent prevalence in 2013-2014 varied between 1.5-5.2% for dairy herds tested with BIO K302 ELISA (Bio-X diagnostics) on BTM in four sampling rounds (Arede et al., 2016). In Finland there were 68 (0.8%) positive dairy herds detected between 2012 to 2018, mainly identified through PCR analysis in routine diagnostics of mastitis milk samples (Vähänikkilä et al., 2019). Ireland has also investigated the prevalence of antibodies to *M. bovis* in BTM and found a high apparent herd prevalence, with 45% of herds being positive (McAloon et al., 2022). In the Irish study the IDScreen® *M. bovis* indirect ELISA was used.

Within-herd prevalence has been evaluated for intramammary infection in four *M. bovis* positive Estonian dairy herds, and the prevalence varied between 3.4% and 12.3% at first sampling of all cows (Timonen et al., 2020). Colostrum has also been studied in *M. bovis* infected herds; 368 samples were tested from 17 herds, 1.9% of the samples tested positive with PCR and all the positive samples came from four herds (Gille et al., 2020). In whole herd sampling in four Australian dairy herds following an *M. bovis* mastitis outbreak, between 0-0.2% of the cows were PCR-positive for *M. bovis* in the milk, despite high seroprevalences (Hazelton et al., 2020b). For calves, the prevalence is often low in beef suckler calves, and high in feedlot calves gathered from different herds (Maunsell et al., 2011). In a study of feedlot calves suffering from a BRD outbreak, the within-pen prevalence of *M. bovis* was 8-100%, median 54%, in cultured samples from transtracheal aspirations (Timsit et al., 2012).

2.2.9 Epidemiology

Mycoplasma bovis is naturally contagious and the transmission within a herd occurs through nose-to-nose contact between cattle, between cows during milking, or by feeding calves infected milk (Calcutt et al., 2018, Fox et al., 2005, Punyapornwithaya et al., 2011). Calves can be experimentally infected through aerosolised culture of *M. bovis* (Kanci et al., 2017), but the importance of aerosols in calf-to-calf transmission in the field is currently unknown. Other routes of transmission, such as contaminated farm environment, fomites, and assisted reproduction are not as well understood. Infected semen has been linked to the introduction of *M. bovis* in two closed dairy herds in Finland (Haapala et al., 2018). Despite the simple structure and sensitivity to dehydration, *M. bovis* can survive in humid conditions and

low temperatures, two weeks in water, and two months in milk and sponges, at 4ºC (Pfützner, 1984). Breeding bulls can also be potential carriers for transmission of mycoplasma both within and between herds (Hazelton et al., 2018a).

Introduction into a naïve herd is usually the result of introducing infected cattle or through contact with infected cattle from another herd. Cows may harbour the infection until stress, such as calving, potentially triggers the onset of a contagious disease (Nicholas et al., 2016). Indeed, asymptomatic carriers are an important factor in the spread of *M. bovis.* Such chronic infections are suspected to persist sub-clinically for up to several years and promote continued circulation and outbreaks in the herd both in calves and cows (Maunsell et al., 2011). Intermittent shedding presents a challenge for accurate diagnosis at the individual animal level and for biosecurity purposes. Therefore, using an ELISA to detect previously exposed animals, and potential carriers has a better sensitivity in finding herds and animals at risk of transmitting the infection (Hazelton et al., 2018b, Wawegama et al., 2016). There is some evidence that clearance of infection is possible, as it has been observed both in infected dairy cows and herds. Dairy cows were followed by culturing samples from milk and mucosal swabs, and herds were followed with PCR-analysis on BTM, nasal swabs from calves and mastitis milk (Vähänikkilä et al., 2019, Punyapornwithaya et al., 2010).

A few risk factors for *M. bovis* infection have been identified. These are larger herd size, introduction of cattle, many herd movements, and stress factors related to feed and overcrowding (Thomas et al., 1982, Burnens et al., 1999, Aebi et al., 2015). A recent study in Belgium showed that the use of a breeding bull and absence of a calving pen are also risk factors (Gille et al., 2018). Moreover, in a questionnaire study in *M. bovis* case and control farms experiencing more infectious diseases (rotavirus, ringworm, respiratory diseases, *Str. agalactiae* mastitis) in the herd during the last 6 months, was determined as a risk factor (Haapala et al., 2021).

2.2.10 Economic impacts

According to a UK study, the costs for treatments and production losses for all respiratory diseases in cattle are approximately £54 million, and about 157,000 calves die annually because of pneumonia, leading to additional losses of £99 million. Extrapolating this to the European cattle industry, consisting of 90 million cattle, will give a total loss of 576 million euros per

year (Nicholas and Ayling, 2003). There are a few available reports on the estimated costs of *M. bovis* infection in the US beef and dairy industry. For the beef industry the costs were 32 million US dollars per year due to reduced weight gain and carcass value, and 108 million US dollars per year in the dairy industry due to mastitis (Maunsell et al., 2011). These numbers seem very high but on a national level the estimations vary considerably, and there can be significant economic losses for individual farmers. In a serious *M. bovis* mastitis outbreak in New York, 30-70% of cows were culled in several herds (Gonzalez et al., 1992). Decreased milk production linked to a positive M. bovis test result has significant financial implications for farmers. In a study in Pennsylvania, intramammary infections caused by *Mycoplasma spp.* reduced the milk production in one lactation by 1,500 kg, which is equivalent to 451.63 US dollars (Wilson et al., 1997). Reduced milk yield in subclinical *M. bovis* mastitis can also cause hidden economic losses (Timonen et al., 2017).

In the New Zealand eradication programme, 189,666 cattle have been culled between July 2017 and December 2024, and the compensation payments have been 289 million NZ dollars (Ministry of Primary Industries, 2024).

2.2.11 Treatment and antimicrobial resistance

Mycoplasma bovis is often resistant to antimicrobial treatment, leading to chronic infections, and culling being the only remaining option (Nicholas et al., 2016). Mycoplasmas are naturally resistant to β-lactams due to their lack of a cell wall. The antimicrobial resistance pattern is similar in several European countries with high MIC (Minimum Inhibitory Concentration) values for oxytetracycline, tylosin, tilmicosin and fluoroquinolones (Klein et al., 2019). Currently, there are no clinical breakpoints available to help interpret data or correlate MIC values with in-vivo efficacy. In the Swedish surveillance of antimicrobial resistance, clinical isolates of *M. bovis* (n=80), sampled from the respiratory tract of calves, through either nasal swabs or from lungs at post-mortem examination, in 2020-2023, were tested for antimicrobial susceptibility. These isolates exhibited high MIC values for tetracycline and gamithromycin (a macrolide), and for most of the isolates, the florfenicol MICs exceeded the VetCAST clinical breakpoints for Pasteurella multocida (R > 1 mg/L) and Mannheimia haemolytica (R > 2 mg/L). Enrofloxacin MICs were low for all the tested isolates (Swedres-

Svarm, 2023). The clinical isolates in Sweden have a very similar antimicrobial resistance pattern and belongs to a new predominant clone in northern Europe, first detected in 2011 in Sweden, but also found in Denmark, Finland, and the Netherlands (Tardy et al., 2020).

The preferred antibiotics in Sweden for treatment of pneumonia or mastitis is penicillin, since the common pathogens are usually sensitive to penicillin (Swedres-Svarm, 2023). However, this strategy may be threatened if *M. bovis* spreads widely.

2.2.12 Prevention and Control aspects

A closed herd policy is the most efficient way to prevent the introduction of *M. bovis* into naïve herds (Maunsell et al., 2011). If this is not possible, the recommendation is that purchased animals are tested and quarantined before entering the herd (Maunsell et al., 2011). New Zealand has an ambitious eradication programme for *M. bovis*, which includes culling cattle at infected farms and tracing contact farms. Testing and surveillance are important aspects of the programme and include individual serum antibody ELISA, BTM antibody ELISA, and tonsillar swabs at slaughter for PCR analysis (Marquetoux et al., 2023). An evaluation of New Zealand's control programme indicated that the transmission of M. bovis between farms has decreased, showing progress towards eradication (Jordan et al., 2021).

In Finland there is a voluntary control programme was established in 2013 to decrease the risk of introducing *M. bovis* in relation to animal trade (Haapala et al., 2021). This programme includes regular veterinary herd health visits, routine PCR testing of mastitis milk samples for *M. bovis*, and PCR analysis on nasal swabs from calves and BTM samples twice a year.

To control the infection within a herd, culling or isolating *M. bovis* mastitis cows is suggested, along with raising calves separate from older animals, pasteurising the milk, and applying better hygiene methods (Punyapornwithaya et al., 2012, Aebi et al., 2015). Control measures were evaluated in a Finnish study, and the most important ones were found to be isolation of calves, preventing nose-to-nose contact between calves and older animals, together with testing mastitis milk and culling *M. bovis* positive cows (Haapala et al., 2021).

2.2.13 Mycoplasma bovis vaccine

At present, there are no effective vaccines available for control of diseases caused by *M. bovis* (Perez-Casal et al., 2017). Many efforts have been made, and several research groups have developed experimental vaccines, but without any satisfactory effect (Dudek et al., 2021). Indeed, there are various challenges to overcome within vaccine development, one being the need of an animal challenge model, another being consideration for the contribution of other respiratory pathogens in BRD involving *M. bovis* (Perez-Casal et al., 2017). The use of bioinformatics tools is expected to facilitate the proteomic analysis of the *M. bovis* secretome, potentially identifying novel secreted proteins that could be used both as diagnostic biomarkers and in the development of an effective vaccine for controlling *M. bovis* infections (Dudek et al., 2021).

3. Aims

The overall aim of this research was to obtain knowledge about the epidemiology of *Mycoplasma bovis* infections in Swedish dairy herds.

The specific aims were:

- to investigate the national and regional prevalence of *M. bovis* in Swedish dairy herds,
- to study the effects of *M. bovis* infection on milk yield, and herd health parameters,
- to investigate risk factors for introduction of *M. bovis* to the farm, and biosecurity measures to prevent within-herd spread,
- to describe the dynamics of *M. bovis* infection in dairy herds over time,
- to evaluate herd-level diagnostics of *M. bovis* by comparing antibody tests of BTM and milk from primiparous cows, and
- to evaluate testing strategies to find infected herds.

4. Material and Methods

This section provides a summary of the main issues regarding study populations, data collection, and research methods. A more detailed description is provided in each individual Paper (I-IV). An overview of the performed studies and material is presented in Figures 2 and 3.

4.1 Study population

In Study I, all dairy herds in Sweden were enrolled in a cross-sectional study to investigate the prevalence of *M. bovis* antibodies and bacterial DNA in BTM. A total of 3144 herds were included.

In Studies II, III and IV, dairy herds with more than 70 cows, that were affiliated to the Dairy Herd Improvement (DHI) program, and located in the southern regions were enrolled. The regions included were Halland, Kalmar, Skåne, Västra Götaland and Östergötland. These regions were selected based on prior detections of *M. bovis*, with the aim of including both infected and non-infected herds, except in Study IV where only *M. bovis* exposed herds were included. In Study II, 149 dairy herds were enrolled in a 2-year longitudinal study to investigate the dynamics of *M. bovis* antibodies in milk and herd-level risk factors for introduction.

In Study III, an online questionnaire was sent to the herds enrolled in Study II. The response rate was 80% (n=115), and we believe this will increase our knowledge about *M. bovis* infected herds and their management.

In Study IV, a 2-year longitudinal study, 35 herds with a positive test result for *M. bovis* antibodies in either BTM or individual milk sample in Study II were enrolled. Most of these herds were visited four times and both cows and calves were sampled. The aim was to enhance our knowledge about

patterns of *M. bovis* in cows and calves, and to evaluate the best testing strategy to find infected herds.

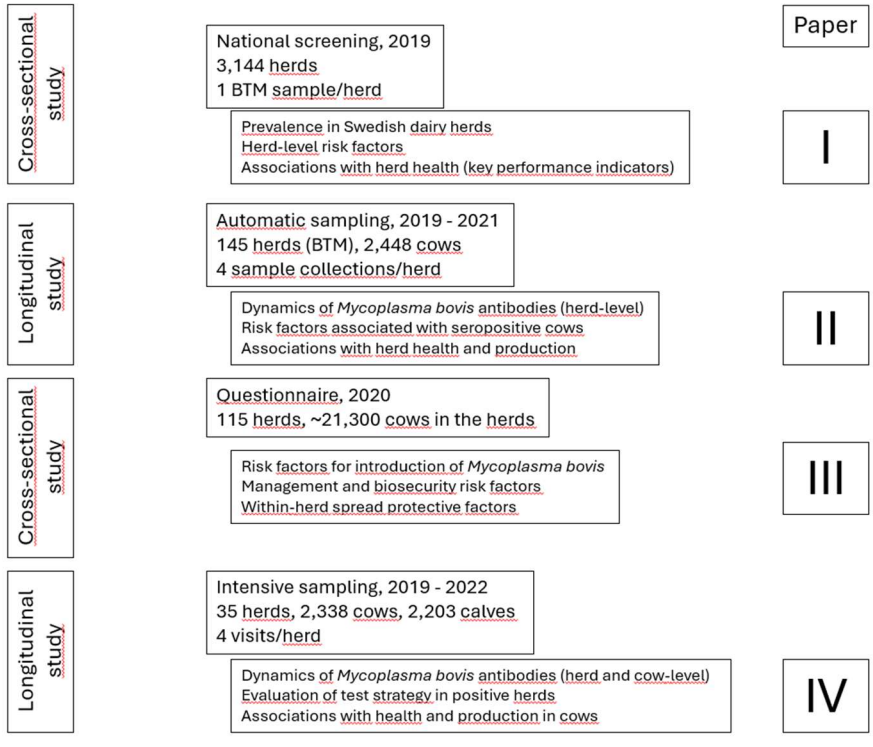

Figure 2. Overview of the studies performed between 2019 and 2022 during this thesis work, with the aim to increase the knowledge of *Mycoplasma bovis* in Swedish dairy herds.

4.2 Sampling

In Studies I-III, the BTM samples were collected at the milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden), in conjunction with the routine milk quality analysis. Individual milk samples from the 3 youngest home-bred primiparous (PP) cows within 6 mo. after calving were collected in the same way for Studies II and III. In addition, in case individual samples could not be collected as described above, the farmers received sampling kits and were asked to sample three PP cows close to calving. This was an unforeseen complication since an inclusion criterion was that the herd

was affiliated to the DHI program (Växa, Sverige) and therefore involved in monthly test milking. The reason for this was because some herds dropped out of the DHI program after enrolment in the study, or were not test milking during the sampling period.

On-farm sampling was carried out in Study IV with a total of 120 herd visits on 35 different farms. This was performed in collaboration with the farmer and the time spent at each visit was around 2 hours. The sampling was done either by the farmer's herd veterinarian or the PhD researcher. At the herd visits both cows and calves were sampled, milk samples from the cows, and blood samples and nasal swabs from the calves. Blood samples were taken from the vein in the neck or the tail.

The study was aiming animals that were at risk of having *M. bovis* infection. Thus, the veterinarian and the farmers were asked to sample cows with a high SCC or a recent mastitis case, 10 PP cows and 10 multiparous (MP) cows. For the calves, the instructions were to select untreated animals, 2-10 months old, with symptoms of pneumonia, otitis, or arthritis. If no such calves existed, healthy calves of the same age were selected instead. The success of the sampling was relying on the farmers' willingness to help with handling of the animals at sampling, and convenience sampling was applied.

At the milk testing laboratory, milk samples were collected in 10-mL test tubes containing 1.5 mg of the preservative agent bronopol (2-bromo-2-nitropropane-1.3-diol). The field samples were collected in sterile tubes. The milk and blood samples were sent in ambient temperature by postal service to the laboratory at the Department of clinical sciences, SLU, Uppsala. The samples were stored at -20ºC until analysis. Nasal swabs were collected with Eswab® (Copan, Murrieta, CA, USA), and sent to the Swedish Veterinary Agency (SVA), Uppsala, and subsequently analysed with PCR at the routine diagnostic laboratory.

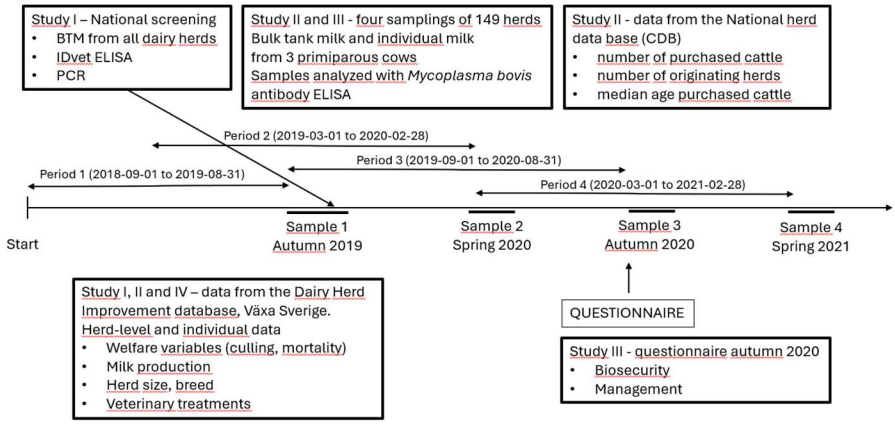

Figure 3. Overview of data collection and sampling from 2018 to 2021 for Studies I-IV included in the thesis work of *Mycoplasma bovis* epidemiology in Swedish dairy herds.

4.3 Data withdrawal

Anonymous aggregated herd-level data on health and produciton were retrieved from the DHI database (Växa Sverige) for the period 1st of November 2018 to 31st of October 2019 (Study I). The farmers enrolled in Studies II, III and IV had given their consent for data withdrawal from the DHI database. In Studies II and IV, herd-level data from the DHI database was used, aggregated in 12-month periods. The start of data withdrawal was 1st of September 2018 and last day was 31st of August 2021. In Study III, the 12-month period, 1st of September 2019 to 31st of August 2020, was included in the analysis. In Study IV, individual cow data on production, treatments, health, and breed was retrieved from the DHI database for the period 1st of September 2018 to 28th of February 2022. In addition, in Study II, data on the number of female cattle older than 24 months, introduction of cattle to the herd including age, and origin of introduced cattle was retrieved from the central register of bovine animals (CDB, Swedish Board of Agriculture) for the period 1st of September 2018 to 31st of August 2021.

4.4 Antibody detection

The milk and blood samples were analysed for the presence of IgG antibodies to *M. bovis*, using a commercially available indirect ELISA (ID Screen®,

IDvet, Grabels, France). The relative amount of antibodies present in the samples was calculated as [sample optical density (OD) – negative control OD]/[positive control OD – negative control OD] × 100 (S/P %). The milk samples were analysed with the overnight incubation protocol and the cut-off for a positive sample was set to S/P% ≥30%, as recommended by the manufacturer. The cut-off for a positive serum sample was set to S/P% ≥60%, as recommended by the manufacturer.

4.5 Bacterial DNA detection

Nasal swabs collected from calves at herd visits in study IV were analysed with PCR as described below. The samples were pooled with 3-4 calves in the same analysis. DNA from nasal swabs was extracted using IndiMag Pathogen kit (Indical Bioscience GmbH, Leipzig, Germany). PCR analysis for *M. bovis* was performed using the primers and probe described by Sachse et al. (Sachse et al., 2010). The reactions consisted of PerfeCTa qPCR Toughmix (Quantabio, Beverly, MA, USA), 500 nM of each primer, 100 nM of probe, and 2 µl DNA template in a total volume of 15 µl. The amplification was performed using ABI 7500 Fast real-time PCR platform (Applied Biosystems, Waltham, MA, USA) and the following temperature profile: 50°C for 10 min, 95°C for 3 min. and 45 cycles of 95°C for 3 sec, and 60°C for 30 sec. Samples with a Ct-value of 40 or below were considered positive for *M. bovis*.

4.6 Information provided to farmers

The farmers were informed via email about their herd test results after each sampling occasion (Study II). To the farmers with test-positive herds, the following information was provided; BTM or an individual sample from a PP cow contained antibodies to *M. bovis*, indicating that the lactating cows have been exposed to the bacteria. The animals may remain asymptomatic while carrying the bacteria. It is crucial to inform prospective buyers of livestock that *M. bovis* is present within the herd. Should you wish to discuss the disease, appropriate management strategies, and measures to minimize risk of transmission, I recommend consulting your herd veterinarian as a first choice. Together with this letter there was also a document with general recommendations from the industry about biosecurity and how to handle *M.*

bovis positive status. The farmers with test-negative herds were provided information about their negative status. In Study IV, the information about test results were sent to both the farmer and the herd veterinarian. Besides the provided general recommendations, the farmer was encouraged to discuss specific strategies with the herd veterinarian.

Participating farmers and their herd veterinarians were also offered contact in a *M. bovis* research project Facebook group. Some results were published in this group and there were a few discussions about the subject and questions about how to handle a positive test result.

4.7 Questionnaire and Biosecurity program

In connection with the autumn sampling in 2020 (Study II), a questionnaire was completed by the farmers of the participating herds. The questionnaire contained questions about herd routines regarding external and internal biosecurity measures and management. In addition to the questionnaire, data about the herd's affiliation and status in the biosecurity program "Smittsäkrad besättning" were retrieved from Växa Sverige. In Study III, association between potential external and internal risk factors including affiliation to the biosecurity program (yes or no) and herd-level seropositivity to *M. bovis* was analysed.

4.8 Test strategy

The probability of identifying positive herds was calculated by evaluating seven different testing strategies across one, two, three, or four occasions. The test strategies from Study IV included: 1) calves only, 2) cows only, 3) cows and calves, 4) BTM only, 5) BTM and calves, 6) BTM and cows, and 7) BTM, cows, and calves. Each herd was classified as either negative (0 = no positive samples) or positive (1 = one or more positive samples) at herd visit 1, based on the results of the respective test strategy. For subsequent herd visits, the test result from previous visits was incorporated into the evaluation. The number of positive herds was then divided by the total number of tested herds at each sampling, and this ratio was defined as the sensitivity of the test strategy in detecting assumed test-positive herds.

To further assess this test strategy, we aimed to investigate how the sensitivity would be affected by testing 3 or 5 cows, as opposed to testing 20

cows. The same principle as above was used for the calculations. The test results were randomly selected from the total of 20 cows sampled at each herd visit. The random selection was completed in Excel, each cow had a number between 1 and 20 and the program was asked to choose 3 or 5 numbers between 1-20. The result from the selected cows was either negative (0=no positive samples) or positive (1=at least one positive samples).

4.9 Statistical methods

Regression analysis was used in all studies. Regression models are designed to test the association between one or more explanatory variables (predictors) and a dependent variable (outcome). In a regression model, we aim to understand how changes in the explanatory variables are related to changes in the dependent variable, and to quantify the strength of that relationship. Studies I and III are cross-sectional studies that measure associations between predictors and outcome at one time-point. Studies II and IV are longitudinal studies with repeated measurements and therefore regression models that account for clustering were used, i.e. several samples in the same herd.

In Study I, we used linear and logistic regression models to examine herd-level differences in health, production, and other herd characteristics, between antibody-positive and antibody-negative herds. The models had two different approaches as we wanted to assess risk factors for being *M. bovis* positive in BTM and the effects of being positive. In the investigation of risk factors, *M. bovis* status was the outcome, and explanatory variables were herd size and region. Since there were no positive herds in the northern regions, the variable region was excluded from the analysis. In the effect analysis *M. bovis* status was an explanatory variable together with other possible confounders, such as breed, herd size, and milk production. The outcome variables were the herd health characteristics, and they were all assigned a separate model.

In Study II, we used a multilevel linear regression model to investigate possible risk factors that could influence *M. bovis* antibody levels in PP cows. We also used multilevel logistic regression models to investigate associations between herd-level antibody status and herd health characteristics. There were 3 levels of antibody status assigned to the herd, 1) negative in both BTM and PP cows, 2) negative BTM and positive PP

cows, or 3) positive BTM and positive, negative, or missing PP cows. Sample and herd were included as random effects, accounting for repeated measurements and within-herd clustering.

In Study III, we used linear and logistic regression models to investigate herd-level factors, obtained in the questionnaire, associated with herd *M. bovis* status. The *M. bovis* status of the herds was defined as positive either in BTM or in PP cows, or negative. The potential risk factors for seropositivity were analysed divided in internal and external biosecurity factors.

In Study IV, we used regression models to evaluate possible differences in individual milk yield, and cow performance between antibody-positive and antibody-negative cows in 305-days lactation period. The models used were multilevel linear regression, and generalised estimation equation (GEE) with exchangeable correlation structure. Both models accounted for clustering within herds and gave a population-average effect as outcome.

Statistical analyses were performed using the statistical software Stata (Stata statistical software: Release 15.1, 17.0 and 18.0; College Station, TX, USA: StataCorp LP.).

5. Results & Discussion

This chapter offers an overview and a general discussion of the results, along with methodological considerations. A more in-depth discussion can be found in each individual paper.

5.1 Prevalence in Swedish dairy herds

The BTM screening in 2019 (Study I) confirmed a low prevalence of *M. bovis* in Swedish dairy herds with an apparent prevalence of 4.8% (n=147) seropositive and 0% PCR-positive. This was the first time a national screening was performed with testing for *M. bovis* antibodies using the IDScreen® indirect ELISA (IDvet, France). Antibody-positive herds can be both herds with an active *M. bovis* infection and herds with a past exposure to the bacterium. In Study IV, antibody positive herds were followed for two years with multiple samples and a positive status in BTM remained over time. Also, most of these herds had a positive *M. bovis* PCR analysis at least once (n=18/22), indicating active infection in the herd. The geographical distribution showed large regional differences in prevalence, ranging from 0% in the north to 20% in the very south (Figure 4). This may be partly explained by the higher density of dairy cattle in the southern regions (Figure 1). A new clone of *M. bovis* was first detected in Sweden in 2011 (region 10, Figure 4), and the spread of the infection has progressed at a relatively slow pace since then.

In a prevalence study of *M. bovis* antibodies in BTM in dairy herds in Denmark there was also a spatial variation across different regions. Certain areas had high risk of herds being test-positive, and others a low risk but regions with a low risk of test-positive herds remained stable throughout the study (Arede et al., 2016). To prevent the spread of a disease, it is imperative

to protect low-risk areas. In Finland, *M. bovis* has been found throughout the country, though most of the detected herds are situated in the mid-west of the country (ETT, Finland 2023). This may be an effect of the geographical distribution and density of cattle herds in Finland.

The low prevalence and slow spread found in Sweden gave incentives and motivation to work on prevention of disease spread and thoughts about a voluntary control program (Hurri et al., 2021).

Within-herd prevalence has not been well studied, in one Estonian herd the within-herd prevalence of *M. bovis* subclinical intramammary infection was 17.2% (Timonen et al., 2017). In Study II, the antibody prevalence among tested cows was 18.4% (n=450). However, in Study IV, wherein positive herds were investigated and a risk-based sampling of cows with high SCC was used, the cow-level antibody prevalence was 56.5% (n=1,052). Prevalence of *M. bovis* within-herd is rather low, ranging between 3.7%-11.0% in clinical mastitis cases and 1.7%-4.7% in colostrum (Timonen et al., 2020).

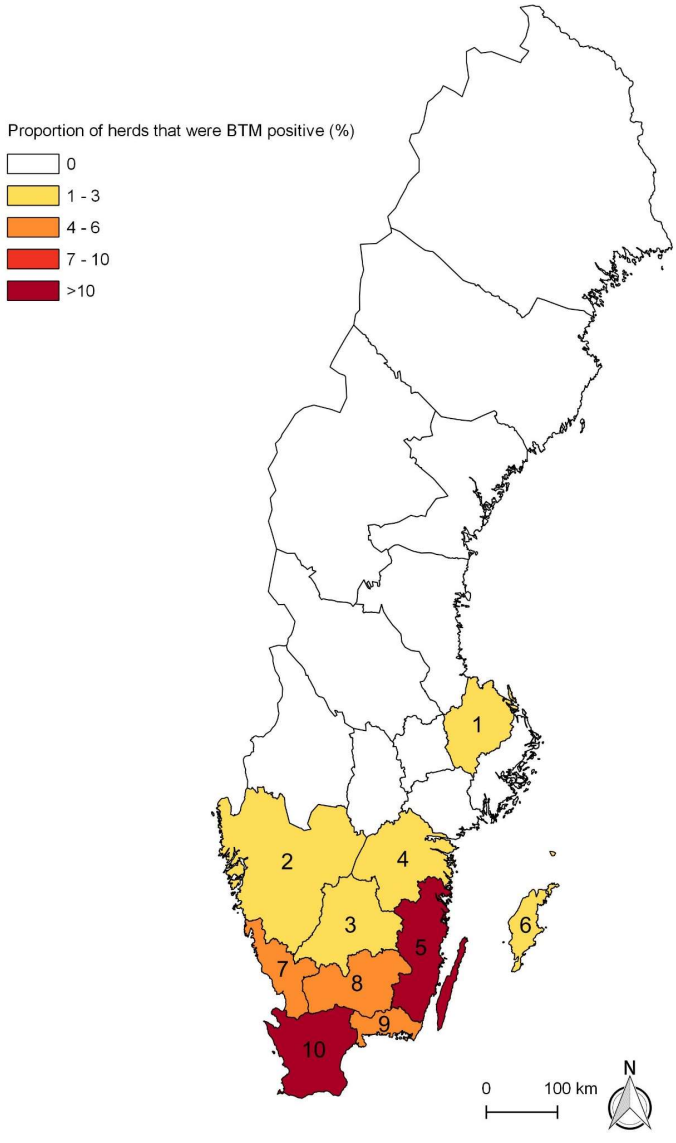

Figure 4. The proportion (%) of herds with antibodies to *Mycoplasma bovis* in bulk tank milk screeening in 2019, for each of the 21 geographic regions in Sweden, 1=Uppsala (1%, n=1), 2=Västra Götaland (3%, n=16), 3=Jönköping (3%, n=10), 4=Östergötland (3%, n=7), 5=Kalmar (13%, n=41), 6=Gotland (3%, n=4), 7=Halland (4%, n=6), 8=Kronoberg (5%, n=6), 9=Blekinge (6%, n=3), 10=Skåne (20%, n=53).

5.2 Health and production

The main findings associated with herd health and production were:

- Herds with antibody positive BTM had a higher late calf mortality (2-6 mo.) and young stock mortality (6-15 mo.) (I).
- Milk production in 305-days lactation was significantly reduced (-404,5 kg milk = 1,33 l/day) in individual cows who were seropositive for *M. bovis* compared to seronegative cows (IV).

These results are unsurprising and have been reported in other studies. The higher mortality in calves could be related to *M. bovis* pneumonia and the fact that pneumonia may be treated several times before a decision is made to euthanise the calf. A calf may also survive the pneumonia but acquire a chronic infection with reduced growth resulting in a "looser" calf that will not be able to perform as a lactating cow (Petersen et al., 2019).

Lower milk yield and higher SCC is associated with subclinical *M. bovis* mastitis (Timonen et al., 2017, Uhaa et al., 1990). Cows were selected for sampling in Study IV based on SCC and udder health, which may introduce bias into the study, although *M. bovis* infection can also occur without any symptoms from the udder. There are limited reports of *M. bovis* mastitis in Swedish dairy herds and we are aware of only one outbreak (Maria Carlson, unpublished, Hurri et al. 2021). Interestingly, there seems to be a subclinical effect with reduced milk yield among Swedish cows, which has an economic impact for the farmer. Another concern relevant to this thesis is whether the higher mortality and reduced milk yield in antibody-positive cows were directly caused by the infection or if the results were confounded by management factors. The measures we have implemented to account for herd factors include maintaining herd size in the analysis and comparing antibody-positive and antibody-negative cows within the same herds.

The health and production data collected from the DHI database (Växa Sverige) were reported by farmers, AI technicians and veterinarians. Farmers differ in their willingness to report, along with the criteria used to decide about culling reason, and whether to treat the cow. An evaluation of the validity of the disease data showed a moderate correspondence between manual herd registers and the DHI database (Mörk et al., 2010). However, the significant outcomes in this study, mortality and milk yield, are objective measures.

5.3 Risk factors

The main findings related to *M. bovis* herd-level seropositivity were:

- Large herd size and location in southern Sweden were found to be associated with antibody positivity in BTM (I).
- Large herd size and introduction of cattle were found to be associated with higher antibody levels in primiparous (PP) cows (II).
- Herd-level antibody-negative status was associated with being affiliated to the biosecurity program "Smittsäkrad besättning" (III).
- Feeding the calves with milk replacer and having weaned calves in groups with more than 15 calves were associated with herd-level antibody positive status. (III)

Herd size has been identified as a risk factor for *M. bovis* infection in BTM in several studies (Thomas et al., 1981, Fox et al., 2003, McAloon et al., 2022). Risk factors associated with *M. bovis* infection in other studies might also be connected to herd size, for example a high average milk production, many animal movements, and purchase of a breeding bull (Aebi et al., 2015, Gille et al., 2018). In Study I, herds with more than 120 cows had 8.8 higher risk of being antibody positive in BTM than smaller herds. Larger herds are likely to have more direct and indirect contacts with other cattle herds and may also have expanded recently. Herd size across Swedish dairy herds has increased throughout the last decades (Swedish Board of Agriculture, 2024). Once an infection is established in a larger herd, it is also more challenging to eliminate, as naive animals are continuously born, susceptible to the infection.

Introducing new animals to the herd is considered the most important risk factor for *M. bovis* transmission between herds (Burnens et al., 1999, Murai and Higuchi, 2019, Fujimoto et al., 2020). Almost half of the herds (Study II) had introduced animals the year before sampling. In another study on Swedish cattle herds, 75% of the herds that had introduced animals did not quarantine the incoming animals (Nöremark et al., 2010). This will further increase the risk of introducing infectious disease and not only *M. bovis*. The association between the introduction of animals and higher antibody levels in PP cows could also be linked to the structure of the Swedish cattle sector,

dairy herds often purchase heifers when expanding the herd (Structure of the Swedish cattle sector, Swedish Board of Agriculture, 2022). However, introduction of cattle in this study (II) could not differ between purchase of new animals and introduction of the farmers' own animals, for example heifers returning to the milking herd after external contract rearing. Information regarding *M. bovis* status of the animals and the originating herd was not available, information that would have been valuable in evaluating this risk factor.

5.3.1 External and internal biosecurity

The biosecurity program "Smittsäkrad besättning" is developed by the Swedish Dairy Farmers´ association Växa Sverige. The goal of "Smittsäkrad besättning" is to contribute to safer biosecurity on farms and to provide government compensation in the event of a salmonella standstill. The program is voluntary and has three levels:

1) The farmer assesses the farm's hygiene and biosecurity risks through an online questionnaire and receives information about biosecurity.
2) A veterinarian visits the farm for control and advisory service at intervals of 18–24 months. Antibodies against *M. bovis* in BTM are analysed twice a year for dairy herds.
3) A veterinarian conducts an advanced biosecurity education for all farm employees.

The questions regarding external biosecurity in our questionnaire (Study III) were inspired by the online questionnaire from the biosecurity program described above. The herds affiliated with the program (n=67/115) had reached level 2 (except 1 herd at level 1) and therefore had a veterinary visit with an evaluation and discussion about biosecurity and hygiene. To prevent disease spread by animal contacts, the animals must be kept separate from cattle not affiliated with the program. There are also other measures that are mandatory in the program, for example protective clothing and boots for visitors, marked entrance for visitors and a hygiene area with soap and water. Following the regulations in the program and likely having an increased awareness about biosecurity could explain the association shown in our study and is very encouraging for this program.

Investigation of the internal biosecurity (Study III) were related to calf housing, colostrum routines, milk feeding routines, milking routines and animal contacts within-herd. The results showed that feeding calves with

milk replacer was more common in *M. bovis* positive herds. Feeding milk replacer is a recommendation for *M. bovis* positive herds to minimize the risk of disease transmission from cow to calf. It was also a common practice in Swedish dairy herds some years ago, almost half of the herds used milk replacer (Pettersson et al., 2001). A cross-sectional study like this is unable to establish cause and effect relationships since it cannot determine which variable came first or whether one variable caused the other. The observed relationship between *M. bovis* positive status and milk replacer might simply be a correlation, not a causal relationship.

The other significant association with *M. bovis* antibody positive status was having weaned calves in groups with more than 15 calves. This could also be a correlation, though there is evidence that increasing animal contacts also raises the risk of bovine respiratory disease with *M. bovis* circulating within the calf group (Ollivett, 2020). Calf groups with 3-8 calves had a lower risk of respiratory disease in an earlier Swedish study (Svensson et al., 2003).

5.3.2 Protecting the calves

In Study IV, we showed that calves could be negative for *M. bovis* antibodies and bacterial DNA in several herds with a high prevalence of *M. bovis* antibodies among the cows. Our hypothesis was that these farms kept their calves separated from the cows or had other measures to accomplish this break in the infection route. Therefore, we conducted an analysis of a subpopulation from study III, comparing herds with positive cows and negative calves (n=10) to herds with positive cows and positive calves (n=11), information from Study IV. Aside from separating the calves from older animals, important *M. bovis* disease prevention measures could include removing the calf from the cow shortly after birth, avoid feeding of high SCC milk to the calves, strengthening the calves' immune system through proper colostrum and milk feeding routines, maintaining low infection pressure by keeping the calves in small groups without mixing, and ensuring good hygiene and a clean environment (Haapala et al., 2021, Gorden and Plummer, 2010, Butler et al., 2000). The most relevant variables from the questionnaire (Study III) are described for herds where we had knowledge about *M. bovis* status in the calf group, negative or positive (Table 1). Three variables were associated with *M. bovis* positive calves (p-value <0.05): feeding the calves high SCC milk, keeping pre-weaned calves in groups with

more than 9 calves, and keeping weaned calves in groups with more than 15 calves. Group size is a risk factor of infectious disease among calves, this was discussed in the previous section 5.3.1. For calves, the risk of transmission with *M. bovis* from cows through infected milk is evident (Maunsell et al., 2012). Although low prevalences of *M. bovis* in subclinical mastitis and colostrum have been shown, giving waste milk to calves should be avoided (Timonen et al, 2020). Feeding calves with high SCC milk was associated with *M. bovis* positive calves in this sub study. This is an important finding, as studies like this are rare, and it strengthens the existing advice. The general recommendation is to give the calves milk replacer or pasteurized milk if *M. bovis* is present in the herd. Having positive calves was also linked to larger herds (median of 285), compared to herds with negative calves (median of 133). Larger herds are at a higher risk of *M. bovis* continuing to circulate among the calves, as newborn calves can acquire the infection from older animals, thereby maintaining the chain of infection within the herd (Timsit et al., 2012, Aebi et al., 2015). Breaking the infection route from cows to calves and between calves is very important in reducing *M. bovis* disease.

Table 1. Univariable associations between *M. bovis* status in calves and management practices analysed with chi-square test, or Fisher's exact test when expected count <5.

Variable	Category	Negative calves (n=10)	Positive calves (n=11)	P-value
Herdsize[a]		133 (median) 60 (min) 730 (max)	285 (median) 40 (min) 700 (max)	0.049
Time after birth with cow and calf together	Removed directly	2	4	0.537
	<12h	1	3	
	12-24h	5	3	
	>1 day	2	1	
Milk replacer	No	5	3	0.284
	Yes	5	8	
Milk with high SCC	No	10	7	0.034
	Yes	0	4	
Antibiotic milk	No	9	10	0.943
	Yes	1	1	
Group pen: Housing of calves during milk-feeding period	Calf barn	4	7	0.385
	With lactating cows	2	0	
	With dry cows/fresh cows/youngstock	2	1	
	Outdoors	1	3	
Calves per group pen during milk-feeding period	<9	8	4	0.044
	>9	2	7	
Weaned calves/group	3 to 8	6	0	0.011
	9 to 15	2	6	
	>15	2	5	
Housing of weaned calves	Separate barn or outside	7	7	0.635
	With lactating cows	0	0	
	With dry or fresh cows	0	2	
	With youngstock	3	2	

[a] Kruskal-Wallis test was used for this continuous variable.

5.4 Study population

The five areas selected as source population in Study II were intended to reflect the target population: dairy herds with >70 cows in areas with a presence of *M. bovis* in Sweden. The source population is adequately representative of the target population. The main consideration is whether the study population reflects the source population since the herds included were not randomly selected which may have influenced the internal validity. The willingness to participate could be affected by prior knowledge about *M. bovis* and an interest in contributing to research, which may result in selection bias. However, since the antibody status of the herds was unknown before sampling occurred and the selection was not influenced by management skills or their absence, we consider this to be a minor issue. The participating herds were larger (median=150 cows) than average Swedish dairy herds, and 21% of the herds had at least one positive BTM sample and 47% had a positive cow sample at least once. The number of *M. bovis* antibody positive herds were higher in our participating herds than regional prevalence in Study I. This was beneficial for the study since our attempt was to gain further knowledge about *M. bovis* positive herds. Therefore, one consideration is that the participating herds likely had more knowledge and experience with *M. bovis* than the average herd.

5.5 Dynamics of Mycoplasma bovis antibodies

5.5.1 Herd-level

The results from the longitudinal Study (II) show that antibodies in BTM are stable over time in most herds, 19 herds (with 3 or 4 samples) had a positive BTM in every sample, and 3 herds had 2 of 2 positive BTM samples. Herd size in these BTM positive herds were in median 236 cows (IQR 96 – 465). This demonstrates that the infection is unlikely to self-clear in large herds and these herds likely have a continuous circulation of *M. bovis* which boost the antibody responses in the cows. Antibodies remain in dairy herds for a long time after clinical symptoms of *M. bovis* have vanished, 1.5 years in Finnish herds (Vähänikkilä et al., 2019) and several months in Dutch herds (Penterman et al., 2022). In comparison, there were 82 antibody-negative herds with a herd size of in median 114 cows (IQR 80-191), indicating that smaller herds may have a lower risk of introducing *M. bovis*, as previously

shown in Study I. Also, there may be unknown factors related to biosecurity and management that may have contributed to the differences between these herds.

Few herds showed changes in *M. bovis* antibody levels in BTM. Five herds had increasing *M. bovis* antibody levels in BTM and three of these showed a marked rise in antibodies between two samplings. Each of these herds also had several positive PP cows, and they were situated in Västra Götaland (n=4) and Halland (n=1). Five herds had decreasing antibody levels in BTM, and all became negative at the fourth sampling. These herds also had either no or only one positive PP cow at the fourth sampling. They were situated in Halland (n=1), Kalmar (n=2) and Skåne (n=2). Interestingly, the herds that became negative were situated in high prevalence regions, suggesting these farms had antibodies that originated from a historic infection. Herd size in these herds was in median 141 cows (IQR 75-261).

There were 34 herds with positive PP cows, where the antibody test results in BTM were negative throughout the study. However, the number of positive PP cows often changed between samplings, for example from all negative to 1 or 2 positive and back to negative again. The median herd size for these herds was 149 cows (IQR 103-220). In these herds there were most likely no active infection with *M. bovis,* and the significance of having a few positive PP cows remains unclear. However, a few positive PP cows could indicate a new infection that may spread, which is important for farmers to monitor. It is recommended to investigate whether these cows are newly introduced to the herd, or have had any external contact with other herds, such as shared pastures or external heifer rearing. There may be key measures that can be taken to prevent the spread of *M. bovis* further in these herds. It is well-established that asymptomatic carrier animals contribute to the transmission of M. bovis (Maunsell et al., 2011), and introduction of new animals is a risk of infection (Burnens et al., 1999). Among the five herds that converted from negative to positive in BTM, four had a few positive PP cows in the previous samplings. One herd, however, went from antibody-negative in both PP cows and BTM to antibody-positive in both PP cows and BTM, indicating a rapid spread of *M. bovis* within the herd.

5.5.2 Animal-level

In Study IV, we followed *M. bovis* antibody positive herds (n=35) for two years, sampling both cows and calves. The median herd size in these herds

was 150 cows (IQR 97-327). The *M. bovis* status of the herds at the start of the study were BTM positive (n=23) or PP cow positive with negative BTM (n=12), from Study II. We observed interesting patterns in these herds, for example five herds had negative calves and positive cows. Moreover, five herds managed to break the infection route to the calves and went from positive calves to negative calves. A useful strategy to reduce transmission and work towards eradicating *M. bovis* in a dairy herd will be to start with protecting the calves. Raising *M. bovis*-free cows by strictly separating the calves immediately after parturition was demonstrated in a dairy farm with *M. bovis*-associated disease (Brys et al., 1992). It has also been applied in Finnish dairy farms and these farms have reached a low-risk status (Haapala et al., 2021)

For the tested cow groups, PP cows and multiparous (MP) cows, most of the herds had a stable pattern with either a high prevalence among the cows (n=18) or a very low prevalence (n=8). In some herds other patterns were discovered, two herds had positive PP cows and negative MP cows, though in both herds, the prevalence in MP cows had increased at the fourth sampling. Also, two herds had negative PP cows and positive MP cows, and this did not change during the study period. Measuring antibodies in individual animals and BTM is an effective tool for monitoring the progress of *M. bovis* reduction within the herd. Within the cow group, one potential tool for preventing disease transmission could be to section the cows into antibody-positive and antibody-negative groups. Separation of positive and negative animals was used with some success at the bull semen production centre in Skara (Gerwins, 2023). Strategies for successful eradication of *M. bovis* in a dairy herd depend on efficient identification and culling of cows with *M. bovis* mastitis, this has been described in two studies (Byrne et al., 1998, Bicknell et al., 1983). Using antibodies to detect infected cows will be cheaper than PCR-analysis or culture. With time and proper strategies, it may be possible to eliminate the infection from the herd while maintaining the animals.

The results (Study IV) indicate that the introduction of *M. bovis* into a dairy herd generally takes place in the cow group or the pregnant heifer group. Introduction of new animals into a dairy herd are usually heifers. In Study III, 55% (n=26/47) of the herds that introduced animals from other herds had purchased heifers for recruitment, supporting the hypothesis that sampling PP cows is a tool for early detection of *M. bovis* introduction in a

herd, as was also shown in two herds in Study IV. In addition, 12 and 15 herds, had introduced lactating cows or a breeding bull, respectively, which will also enter the cow or heifer group.

5.6 Antibody detection in milk samples and BTM

The relationship between antibody status in individual cows and BTM was evaluated in Studies II and IV. The number of positive PP cows (Study II) was associated with antibody S/P% in BTM (Figure 5).

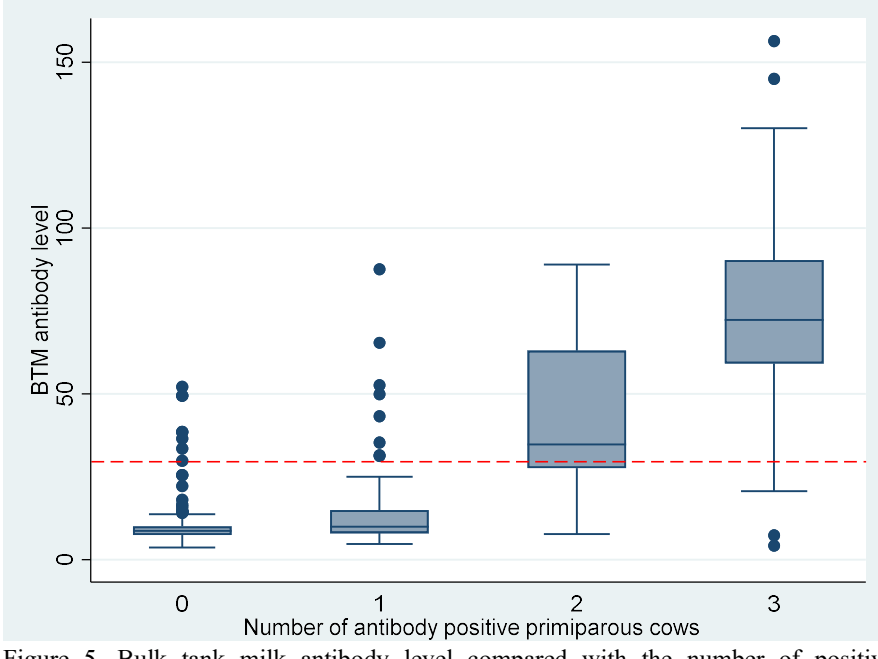

Figure 5. Bulk tank milk antibody level compared with the number of positive primiparous (PP) cows at all sampling occasions. The red line represents the cut-off for the ELISA (S/P ≥30% = antibody positive). Herds with more than 3 sampled PP cows (n=199) were transferred to one of the other categories by dividing the number of positive PP cows with the total number of PP cows sampled. Herds with less than 3 PP cows sampled (n=13) were included in group 0, 1 or 2.

In both studies, the conclusion was that adding samples from individual cows increased the chance of finding infected herds with around 50%. In Study II, almost half (n=67/149) of the herds were positive in PP cows at least once,

compared with 32 herds positive at least once in BTM (Figure 6). The best test strategy is discussed in Chapter 5.7.

The indirect IDScreen® ELISA has a high sensitivity (92.5%-93.5%) which is useful when screening for *M. bovis* at herd-level (Veldhuis et al., 2023, Andersson et al., 2019). Sampling a few individual cows can provide a good indication of herd status, although it does not distinguish between active infection and past exposure to *M. bovis* (Petersen et al., 2020). The specificity (98.6%-99.3%) is also high in this ELISA which will result in few false positives, but not zero (Veldhuis et al., 2023, Andersson et al., 2019). The accuracy of the IDvet ELISA was also evaluated in analyses using antibody test results from surveillance of beef herds in New Zealand, a population with a very low prevalence. There was a high specificity (99.9%) and moderate sensitivity (66.0%) for detecting exposure to *M. bovis* using the IDvet ELISA in these very low prevalence herds (Marquetoux et al., 2023). The low-prevalence herds in Study IV, with only a few positive cows and negative BTM, maintained this status for two years. This might indicate that these herds were not infected with *M. bovis,* and the positive results could be false positives, although the high specificity (99.9%) makes this unlikely. However, these herds may need to remain vigilant and place extra emphasis on biosecurity in case this is a sign of a new infection in the herd. While antibodies to *M. bovis* indicate an immune response to the infection, they do not provide protection against disease (Maunsell and Chase, 2019, Pardon et al., 2015, Nicholas and Ayling, 2003). The complex nature of *M. bovis* infections, including its ability to evade immune defences (Rosengarten et al., 2000), means that animals with antibodies may still harbour bacteria or become re-infected.

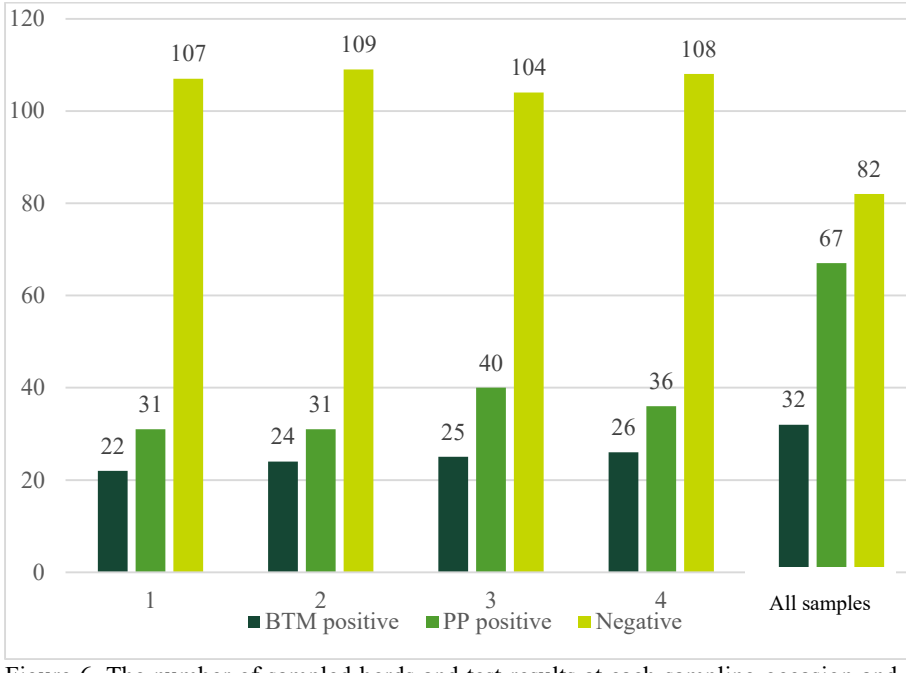

Figure 6. The number of sampled herds and test results at each sampling occasion and for all four samplings together.

5.7 Test strategy and costs

The results from the seven test strategies in Study IV showed that test sensitivity increased with the number of testing occasions, and all strategies including individual cow tests resulted in a higher sensitivity. To further investigate this, we evaluated the test sensitivity for 3 or 5 cows in comparison with 20 cows. In the biosecurity testing program "FriskKo" (Växa Sverige), 3-5 PP cows are sampled and tested for *M. bovis* antibodies twice per year. The test sensitivity was reduced when sampling fewer cows, 5 cows were better than 3 cows, and adding a few cows resulted in a higher sensitivity than BTM samples only. At the third sampling the test sensitivity was 81.5% and 85.2% for 3 and 5 cows, respectively (Table 2, Figure 7).

Adding a test on individual samples is straightforward if the herd participates in monthly test milking, and the cost in these cases is low. Our samples were analysed separately, but pooling 3-5 samples is likely sensitive enough to detect positive herds (personal message, Viktor Ahlberg, SVA). Calculating the costs of running a control program will, in addition to costs

for laboratory analysis, include administrative expenses, sampling costs, and the farmers' costs associated with restrictions on cattle trade and separate transportation of animals. These costs should be compared with the costs of disease (Study I and IV). Maintaining healthy animals will be profitable (Hultgren et al., 2008).

Table 2. Probability (%) of finding *M. bovis* antibody positive herds with different testing strategies.

Sample	BTM	20 cows	3 cows	5 cows	BTM and 20 cows	BTM and 3 cows	BTM and 5 cows
1	60.0%	77.1%	68.6%	71.4%	77.1%	71.4%	71.4%
2	67.7%	90.3%	71.0%	77.4%	90.3%	74.2%	77.4%
3	77.8%	96.3%	74.1%	85.2%	96.3%	81.5%	85.2%
4	76.9%	100.0%	80.8%	88.5%	100.0%	84.6%	88.5%

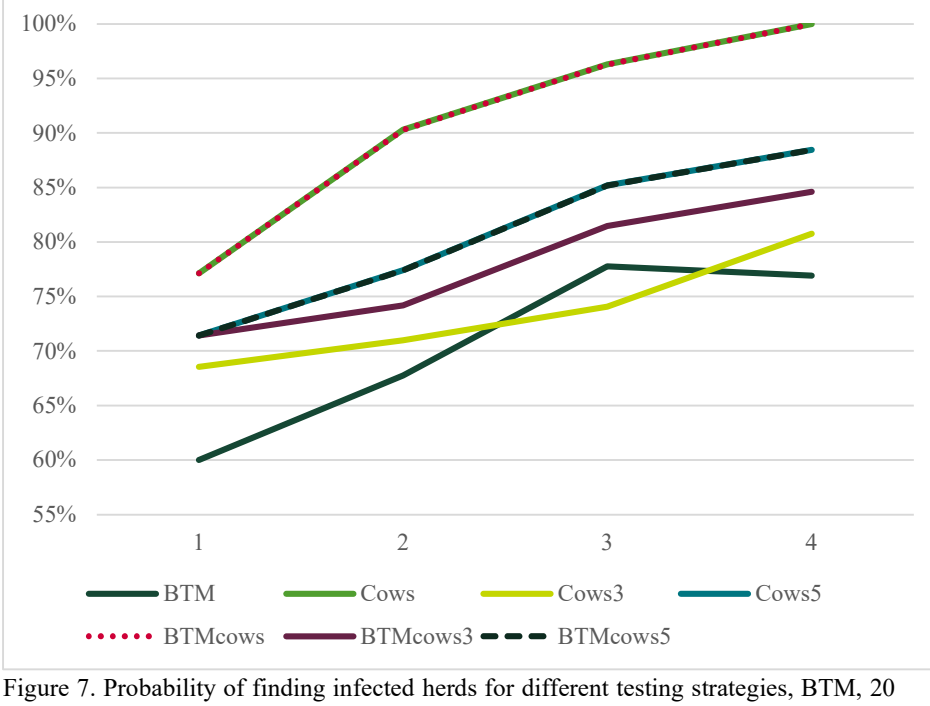

Figure 7. Probability of finding infected herds for different testing strategies, BTM, 20 cows, 5 cows, or 3 cows, at 1, 2, 3, or 4 sampling occasions.

5.8 Prospects for control

The findings presented in this thesis suggest that introduction of cattle, without knowledge of the animals *M. bovis* status, and the level of biosecurity are the most important factors in the transmission of *M. bovis* between farms. We have also reported that testing for *M. bovis* antibodies is cost-effective and easily done by sampling BTM and a few individual PP cows. Thus, it seems clear that there is a possibility of controlling the spread of *M. bovis* in a cost-effective and practical way via increased biosecurity and herd-level testing.

In Study I, we reported that most of the herds were free from *M. bovis* in BTM, demonstrating that prospects for control in Sweden were still very good. There has not been any new national screening, but within the biosecurity program "Smittsäkrad besättning", approximately 1500 dairy herds have been tested for *M. bovis* antibodies in BTM twice per year in 2023 and 2024. In the most recent sampling (Aug.–Sept. 2024), 9% (n=132/1467) of the tested dairy herds were positive (personal message, Julia Österberg, Växa). The positive herds were situated in the south of Sweden. In 2019, 7% (n=147/2213) of the herds were antibody positive in the southern regions. Therefore, it appears that *M. bovis* prevalence has not been rising among Swedish dairy herds, and there is evidence of progress in the attempts to prevent disease spread. This work to prevent *M. bovis* transmission had a kick-off meeting in October 2019, which also marks the start of this thesis work, though they are not connected. At the meeting, the dairy and beef industry, authorities, veterinarians, and advising companies agreed that a joint effort was needed to stop the spread of *M. bovis*. This agreement has then been carried out by the organisations Farm and Animal Health and Växa Sverige and has included work with an increased awareness and knowledge gathering. To further increase the knowledge about *M. bovis* in Sweden and motivate control strategies, calculations of disease spread scenarios in Sweden were conducted at the Swedish Veterinary Agency (SVA) in 2020. These calculations indicated that the number of *M. bovis* infected herds was expected to increase rapidly in the coming years unless preventive measures were implemented (Hurri et al., 2021). In the same evaluation, the effects of a voluntary control program for dairy herds were also investigated (Cecilia Hultén, SVA, unpublished). The program would include testing and restrictions on livestock trade. The conclusion was that a high participation

rate, 90% of the dairy herds, would result in a gradual reduction in disease prevalence over time.

The *M. bovis* testing of dairy herds was further developed in 2021, implementing the results from study II in a biosecurity testing program "FriskKo" (Växa Sverige). Antibody testing of BTM and 3-5 PP cows was incorporated into this program to enhance the sensitivity of detecting *M. bovis*-infected herds compared to PCR analysis. A dairy herd with a *M. bovis* positive sample was contacted and informed about the disease and offered help in handling it. The farmer pays €357.23 (3980 SEK) for one year (2025) for testing the herd in "FriskKo". Currently, around 10% of the dairy herds have signed up, a number that could be increased. Controlling the spread of *M. bovis* would be possible with antibody testing and only introducing *M. bovis* free animals from free herds. Analysing antibodies in BTM and a few cows will in many cases be sufficient in uncovering the status of the herd. However, in the case of a negative herd status, further investigation is needed to be sure of a free (low risk) herd status. Sampling of the animals for sale will be important, and sampling of more cows, calves and youngstock is also possible. In addition, examining recent introduction of animals and interactions with animals from other herds. A future control program might need to eventually include all cattle herds involved in animal trade, though control for dairy herds would be a promising start. For suckler herds and fattening herds the costs for testing are higher, though it would be highly valuable for the calf health if the calf-buying herds were able to buy *M. bovis* free calves.

We believe that it is not consistent with a sustainable healthy cattle population to accept outbreaks of *M. bovis* among calves when there is a possibility of controlling the disease spread. Increased biosecurity would not only prevent the spread of *M. bovis*, but also make Swedish cattle farms better prepared for future epizootics and prevent the spread of zoonotic infections as well as other contagious infections. A reduced prevalence of *M. bovis* in dairy herds would benefit the farmer economically, enhance animal welfare, and reduce the use of antibiotics, both in dairy and fattening herds.

6. Conclusions

- Sweden has a low prevalence of *M. bovis* with around 90% free dairy herds, and these free herds are important to protect.

- To find the infection early it is necessary to sample individual cows.

- Health effects are observed in Swedish dairy herds. Increased calf and young stock mortality, and reduced milk production at cow level. This has financial consequences for the farmers.

- Risk factors for introduction of disease are herd size and purchase of cattle. Being affiliated to the biosecurity program "Smittsäkrad besättning" could be beneficial since it was associated with negative *M. bovis* status.

- It is possible to protect calves from *M. bovis* infection despite antibody positive cows in the herd. This is likely due to management and biosecurity routines, separating the calves from the cows.

- The results of this thesis suggest that a control program for *M. bovis* is feasible and beneficial for Swedish dairy farmers.

- Preventing the spread of *M. bovis* among dairy herds will also protect calves in fattening herds from disease and the entire cattle sector will benefit from healthier animals.

7. Future perspectives

Based on the insights and the increased knowledge gained from the studies in this thesis, some specific areas suggested for further research are:

- Local spread between herds seems to be an important factor in *M. bovis* epidemiology. The prevalence of *M. bovis* in Sweden is highest in the very south where we had the first cases and then it has spread slowly to other regions. We also saw a tendency of clustering of antibody positive herds in study II though we lacked the data to investigate this further. It would be an interesting study to look at risk factors for having a *M. bovis* positive herd nearby and to include antibody status in a study of cattle movements. There is already ongoing research as a continuation of this project (MycoModel - Stop the spread of *Mycoplasma Bovis* and protect free herds). The MycoModel project is working with disease spread modelling, how animal movements are connected to the spread of *M. bovis*. A disease spread model will be developed where various control strategies can be tested. The project also includes a survey to investigate farmers' attitudes about control measures to prevent *M. bovis* introduction to the herd.

- Studies examining the attitudes of farmers and veterinarians towards new information on biosecurity and disease prevention would be valuable. MycoModel is a promising starting point and will contribute to further knowledge in this field. To achieve widespread adoption of research-based recommendations, it is essential to develop a strategy for effectively communicating with farmers and veterinarians, ensuring their continued engagement throughout the

research process and that the findings are relevant to the end users. It will facilitate the implementation of outcomes in the herds.

- A study to investigate the possibility of eradicating *M. bovis* from an infected dairy herd would increase our knowledge about effective control measures. A good starting point will be to break the infection route from cows to calves and grouping the cows according to antibody levels. These measures will reduce the transmission of the infection within the herd, but will they be sufficient? What additional steps are needed to eliminate the infection from a dairy herd?

- A study on the performance of bull calves from *M. bovis* free or *M. bovis* antibody positive dairy herds at the fattening herd level would be of interest for the cattle meat production sector. This study would include costs for separate transportation, testing, and the benefits of healthier calves. It could also encourage farmers to pay a premium for bull calves from *M. bovis*-free herds. Additionally, it could prompt structural changes in livestock trading, particularly regarding calves.

- Another way to increase the epidemiological knowledge about *M. bovis* would be to use molecular analysis, i.e. whole genome sequencing, to study contact tracing and transmission pathways.

- The development of an effective vaccine against *M. bovis* infection would be highly advantageous. Such a vaccine could be used in conjunction with biosecurity measures to reduce infection pressure, particularly on larger farms that routinely introduce new animals.

- Routine collection of BTM and milk samples from primiparous cows is already in place. These samples could be used in a wider control program for all dairy herds or the herds in *M. bovis* positive regions, to prevent introduction of *M. bovis* to new herds. It will give possibilities of continued epidemiological studies and to control the infection. This could be done in alignment with other infectious diseases like bovine coronavirus and bovine respiratory syncytial virus.

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Popular science summary

In the last 15 years, an increasing number of cattle herds in Sweden have been affected by *Mycoplasma (M.) bovis*, primarily in the form of pneumonia in calves in fattening herds. In addition to pneumonia, *M. bovis* can also cause arthritis and otitis media in calves, as well as mastitis in cows. The symptoms can range from mild to severe and can affect individual animals or entire groups. Certain animals can also become asymptomatic carriers and spread the disease when moved to another herd. Calves with pneumonia are usually treated with antibiotics, but the infection can often become chronic, leading to the calf being euthanised. For cows with mastitis, there is no effective treatment, and there are currently no effective vaccines on the market.

M. bovis is widespread worldwide and is considered endemic in many countries. The prevalence of antibodies in bulk tank milk has been analysed in several countries. In the Netherlands, 17% of dairy farms were positive, in Ireland 45%, and in Denmark between 1.5% and 5.2%. In New Zealand, a ten-year eradication program is underway to eliminate *M. bovis* there. *M. bovis* primarily spreads through direct contact between animals, via nose-to-nose contact, during milking, or via infected milk to calves. The bacterium lacks a cell wall and is sensitive to desiccation but can survive for several weeks in moist and cold environments. Transmission through equipment and visitors is uncertain, but the bacterium is likely transmitted over short distances. The most important source of infection, however, is the introduction of live animals and contact between herds.

The overall aim of this thesis was to gain better knowledge about the epidemiology and prevalence of *M. bovis* infections in Swedish dairy herds.

In the first study, a bulk tank milk survey was conducted across all of Sweden's dairy herds (n=3,144). The prevalence of antibody-positive herds was 4.8% overall. There was a large regional variation; in the northern regions, all herds were negative, whilst the prevalence in Götaland varied between 3 and 20%.

The remaining three studies included dairy farms (n=149) with more than 70 cows from Halland, Kalmar, Skåne, Västra Götaland, and Östergötland. Bulk tank milk samples and milk samples from three first-calvers were collected four times, with six-month intervals, over two years (2019–2021). On a smaller proportion of the farms (n=35), farm visits were conducted with the collection of bulk tank milk samples, milk samples from both first-calvers and older cows, as well as blood and nasal swab samples from calves/young stock. The samples were then analysed for antibodies. Antibodies persist for a long time, from months to years, although there is uncertainty about their exact longevity. A herd with antibodies against *M. bovis* may have either an ongoing infection or a historical infection. The herd owners were informed about the herd's status after each sampling, and they also received information on how to protect their herd from infection and manage the infection within the farm.

In the first and fourth sub-studies, the relationship between antibody status and health/production at the herd and in individuals was examined. Bulk tank milk-positive herds had higher calf and young stock mortality (2–15 months) compared to negative herds. At the individual level, we found that antibody-positive cows produced 404 kg less milk during a 305-day lactation compared to negative cows.

In the first three studies, risk factors for the introduction of *M. bovis* were identified by comparing herd size, animal introduction, and biosecurity practices. Larger herds and the introduction of animals were significant risk factors for positive antibody status. Affiliation to the biosecurity program "Smittsäkrad besättning" was a protective factor. Our findings provide increased evidence that the introduction of animals and contact with other herds are the primary routes of transmission of *M. bovis* between herds.

In the second and fourth sub-studies, the antibody dynamics over two years were examined. Bulk tank milk-positive herds remained consistently positive, whilst herds with positive first-calvers often changed status. Sampling a small number of first-calvers increased the chance of detecting infected herds by 50%.

In the fourth sub-study, antibody status in different animal groups, including both cows and calves, was followed. In several herds, calves were antibody-negative whilst most cows were antibody-positive. This leads us to believe that the infection is introduced into the cow group. Calves could stay free from infection, and internal transmission was interrupted in a couple of herds. Several herds managed to reduce infection in the calves and moved from a high proportion of positive calves to completely negative calves. The fourth study also included an analysis of testing strategies. We found that it was cost-effective and increased the chance of detecting antibody-positive herds if samples from individual cows were added to the BTM samples.

In conclusion, based on the results of this doctoral thesis, we believe that a cost-effective and practically feasible voluntary control program against *M. bovis* can be organised through increased biosecurity and sampling. We argue that increased knowledge of a herd's antibody status, along with information about the infection, will lead to greater interest in biosecurity and higher receptivity to new routines among all stakeholders. Reducing the spread of *M. bovis* is particularly important for the youngest and most vulnerable animals, the calves. Improved calf health is crucial for growth and production and essential for herds that buy calves.

Enhanced biosecurity in Swedish dairy herds brings several benefits. It not only prevents the spread of *M. bovis* but also protects the herds from other infectious diseases, both established and new ones. Fewer infections would improve animal welfare, provide economic benefits for the farm owners, and reduce the use of antibiotics.

Populärvetenskaplig sammanfattning

De senaste 15 åren har ett ökande antal nötkreatursbesättningar i Sverige drabbats av *Mycoplasma (M.) bovis*, framför allt i form av lunginflammationer hos kalvar i ungnötsbesättningar. Förutom lunginflammation kan *M. bovis* även orsaka ledinflammation och mellanöreinflammation hos kalvar samt juverinflammation hos kor. Symtomen kan variera från lindriga till allvarliga och kan drabba enstaka djur eller hela djurgrupper. En del djur kan dessutom bli symtomlösa smittbärare och sprida sjukdomen vidare vid flytt till en annan besättning. Kalvar med lunginflammation behandlas vanligtvis med antibiotika, men infektionen kan bli kronisk, vilket kan leda till att kalven måste avlivas. För kor med juverinflammation finns ingen effektiv behandling och det finns för närvarande heller inga effektiva vacciner på marknaden.

M. bovis är spridd över hela världen och anses vara endemisk i många länder. Förekomsten av antikroppar i tankmjölk har analyserats i ett flera länder. I Nederländerna var 17% av mjölkgårdarna positiva, på Irland 45% och i Danmark mellan 1,5% och 5,2%. På Nya Zeeland pågår ett tioårigt bekämpningsprogram för att utrota *M. bovis* från landet. *M. bovis* sprids främst genom direktkontakt mellan djur, via noskontakt, under mjölkning eller via infekterad mjölk till kalvar. Bakterien saknar cellvägg och är känslig för uttorkning, men kan överleva flera veckor i fuktiga och kalla miljöer. Betydelsen av smittspridning genom redskap och besökare är osäker, men bakterien kan troligen överföras korta sträckor. Den största smittkällan är dock införsel av levande djur och djurkontakter mellan besättningar.

Avhandlingens övergripande syfte var att få bättre kunskap om *M. bovis* infektioners epidemiologi och förekomst i svenska mjölkbesättningar och hur man kostnadseffektivt kan testa om smittan finns i en besättning.

I den första studien genomfördes en tankmjölksundersökning av alla Sveriges mjölkbesättningar (n=3 144). Förekomsten av antikroppspositiva gårdar var totalt 4,8 procent. Det fanns en stor regional variation, i de norra regionerna var alla gårdar negativa medan förekomsten i Götaland varierade mellan 3 och 20 procent.

I de övriga tre studierna ingick mjölkgårdar (n=149) med mer än 70 kor från Halland, Kalmar, Skåne, Västra Götaland och Östergötland. Från dessa gårdar samlade vi in tankmjölksprover och mjölkprover från tre förstakalvare fyra gånger med sex månaders intervall under två år (2019–2021). På en mindre andel av gårdarna (n=35) gjordes gårdsbesök med insamling av tankmjölksprov, mjölkprov från både förstakalvare och äldre kor samt blodprover och nässvabbsprover från kalvar/ungdjur. Proverna analyserades för antikroppar. Antikroppar finns kvar under lång tid, månader till år, även om osäkerhet råder kring exakt hur länge. En besättning med antikroppar mot *M. bovis* kan ha antingen en pågående infektion eller ha haft en infektion relativt nyligen. Djurägarna informerades om status i besättningen efter varje provtagning, de fick också information om hur de kan skydda sin besättning mot smitta samt hur de kan hantera smittan inom gården.

I den första och fjärde delstudien undersöktes sambandet mellan antikroppsstatus och hälsa/produktion på besättningsnivå och individnivå. De tankmjölkspositiva gårdarna hade högre kalv- och ungdjursdödlighet (2–15 månaders ålder) jämfört med negativa gårdar. På individnivå fann vi att antikroppspositiva kor producerade 404 kg mindre mjölk under en 305-dagars laktation än negativa kor.

I de tre första studierna identifierades riskfaktorer för introduktion av *M. bovis* genom jämförelse av besättningsstorlek, införsel av djur och rutiner för biosäkerhet. Större gårdar och införsel av djur var signifikanta riskfaktorer för positiv antikroppsstatus. Anslutning till smittskyddsprogrammet Smittsäkrad besättning var en skyddande faktor. Våra fynd ger ökad evidens för att införsel av djur och kontakt med andra besättningar är de viktigaste smittspridningsvägarna för *M. bovis*.

I den andra och fjärde delstudien undersökte vi infektionsdynamiken över två år. Vi fann att tankmjölkspositiva gårdar var stabilt positiva, medan gårdarna med enbart positiva förstakalvare ofta ändrade status. Provtagning av ett fåtal förstakalvare ökade chansen att hitta infekterade gårdar med 50 procent.

I den fjärde delstudien följde vi även antikroppsstatus för olika djurgrupper både kor och kalvar. Här kunde vi se att antikroppar mot *M. bovis* var vanligare hos korna. I flera besättningar var kalvarna antikroppsnegativa samtidigt som de flesta korna hade antikroppar. Detta tyder på att smittan introduceras i kogruppen. Vi såg också att den interna smittspridningen till kalvar kunde brytas. Flera gårdar lyckades minska smittan hos kalvarna och gick från en hög andel positiva kalvar till helt negativa kalvar. Den fjärde studien innehöll också en analys av provtagningsstrategi. Vi fann att det var kostnadseffektivt och ökade chansen att hitta antikroppspositiva gårdar om prover från individuella kor togs utöver tankmjölksproverna.

Sammanfattningsvis, baserat på resultaten i denna doktorsavhandling, anser vi att ett kostnadseffektivt och praktiskt genomförbart frivilligt kontrollprogram mot *M. bovis* kan organiseras genom ökat smittskydd och provtagning. Vi tror att ökad kunskap om besättningens antikroppsstatus, i kombination med information om infektionen, kan leda till större intresse för smittskydd och en högre mottaglighet för nya rutiner bland alla aktörer. En minskad spridning av *M. bovis* är särskilt viktig för de yngsta och mest känsliga djuren, kalvarna. En förbättrad kalvhälsa har stor betydelse för tillväxt och produktion under nötkreaturets liv och är avgörande för besättningar som köper in kalvar.

Ett stärkt smittskydd i svenska mjölkbesättningar medför flera fördelar. Det förhindrar inte bara spridningen av *M. bovis*, utan skyddar även besättningarna mot andra infektionssjukdomar, både etablerade och nya. Färre infektioner skulle förbättra djurvälfärden, ha ekonomiska fördelar för djurägare och minska användningen av antibiotika.

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Herd-level prevalence of *Mycoplasma bovis* in Swedish dairy herds determined by antibody ELISA and PCR on bulk tank milk and herd characteristics associated with seropositivity

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ABSTRACT

Mycoplasma bovis is an important pathogen causing pneumonia, mastitis, and arthritis in cattle, leading to reduced animal welfare and economic losses worldwide. In this cross-sectional study, we investigated the prevalence of *M. bovis* in bulk tank milk (BTM) and herd characteristics associated with a positive antibody test result in Swedish dairy herds. Bulk tank milk samples from all Swedish dairy herds (n = 3,144) were collected and analyzed with ID Screen antibody ELISA and PCR. Information on herd characteristics was collected from the national Dairy Herd Improvement database. To identify herd characteristics associated with the presence of antibodies in BTM, logistic regression was used in 4 different models. The apparent herd-level prevalence of *M. bovis* infection based on antibodies in BTM was 4.8%, with large regional differences ranging from 0 to 20%. None of the BTM samples was positive by PCR. All the antibody-positive herds were situated in the south of Sweden. The logistic regression model showed that larger herds had higher odds of detectable antibodies in BTM (herd size >120 cows, odds ratio = 8.8). An association was also found between antibodies in BTM and both a higher late calf mortality (2–6 mo) and a higher young stock mortality (6–15 mo). This study showed a clear regional difference in the apparent prevalence of *M. bovis* infection based on antibodies. The relatively low prevalence of *M. bovis* in Sweden is a strong motivator for the cattle industry to take steps to prevent further spread of the infection. It is essential that the *M. bovis* status of free herds be known, and the regional differences shown in this study suggest that testing is highly recommended when live cattle from high-prevalence areas are being introduced into herds. We do not recommend using PCR on BTM to detect infected herds, owing to the low detection frequency in this study.

Key words: bulk tank milk, ELISA, herd size, *Mycoplasma bovis*

INTRODUCTION

Mycoplasma bovis is an emerging pathogen that causes severe disease in cattle in many countries (Maunsell et al., 2011), most often pneumonia, mastitis, arthritis, and middle ear infection (Nicholas and Ayling, 2003). During the past 10 years, *M. bovis* has been detected in new areas: first in Sweden in 2011 (Ericsson Unnerstad et al., 2012), in Finland in 2012 (Vähänikkilä et al., 2019), and in New Zealand in 2017 (Dudek et al., 2020). This bacterium is naturally resistant to penicillin and infections often fail to respond to broad-spectrum antibiotics, resulting in chronic disease that compromises animal welfare and causes great economic loss for the cattle industry (Nicholas and Ayling, 2003). Some risk factors that have been identified for dairy herds with *M. bovis* include large herd size (Thomas et al., 1981); purchase of animals (Burnens et al., 1999); forestripping, high milk production, and within herd movements (Aebi et al., 2015); and use of a breeding bull and lack of calving pens (Gille et al., 2018). Elimination of *M. bovis* from infected herds is believed to be difficult or even impossible, although raising calves separately from older animals has been suggested (Pfützner and Sachse, 1996; Aebi et al., 2015). In extreme situations, culling all the animals may be done (Pothmann et al., 2015). In cases of *M. bovis* mastitis, the recommendations are to separate and cull infected animals instead of attempting treatment (Fox et al., 2005; Nicholas et al., 2016).

Preventing infection both on the herd and the animal levels is the key to success. Given the lack of an effective commercial vaccine (Perez-Casal et al., 2017), the core of prevention needs to be based on herd diagnostics and the identification and elimination of epidemiological

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risk factors for *M. bovis* infection. Attempts to control the disease are ongoing in both Finland (voluntary control program) and New Zealand (eradication) (Dudek et al., 2020).

Diagnosing *M. bovis* is a challenge both on the individual animal level and the herd level. Culturing, which is laborious, time-consuming, and costly, used to be the only available method for *M. bovis* detection (Sachse et al., 1993). With the development of commercial antibody ELISA and PCR tests, screening of animals has become feasible, and the presence of *M. bovis* in a herd can more easily be detected (Cai et al., 2005; Wawegama et al., 2014; Andersson et al., 2019). The advantages of antibody tests are that they are simple, inexpensive, and rapid; in addition, they also detect previous infection, which is useful in identifying herds for further investigation. A newly developed antibody ELISA, the ID Screen (IDvet), has a diagnostic sensitivity and specificity of 95.7 and 100%, respectively, according to the manufacturer. The studies underlying these figures, however, have not been published. In an interlaboratory comparison between BIO K302 ELISA (Bio-X Diagnostics) and the ID Screen ELISA, applying a latent class analysis, the sensitivity and specificity were high for the ID Screen—sensitivity, 93.5%, (95% posterior credibility interval 0.898–0.965) and specificity, 98.6%, (95% posterior credibility interval 0.976–0.994)—and statistically significantly lower for the BIO K302 (Andersson et al., 2019). An earlier study using an in-house version of the ID Screen method showed similar results (sensitivity, 94.3%; specificity, 94.4%) (Wawegama et al., 2016). In a study by Petersen et al. (2020), the ID Screen was evaluated under field conditions, and the observed correlation between serum and milk values showed that milk samples could replace serum samples for antibody measurement. Individual cows showed high levels of antibodies, which is encouraging for use of the test on bulk tank milk (**BTM**) samples as well (Petersen et al., 2020). A positive correlation between *M. bovis* antibodies in BTM and in sera from individual cows was shown in a limited number of herds (Vähänikkilä et al., 2019), using an in-house ELISA with the same antigen (Wawegama et al., 2014). To our knowledge, our study is the first time that the ID Screen ELISA has been used in a nation-wide study of *M. bovis* prevalence in dairy herds. Multiple types of PCR are being used in different laboratories, including both in-house and commercial ones, with the majority being real-time PCR (Wisselink et al., 2019). The PCR testing has a high analytical sensitivity and can detect bacterial loads between 10 and 240 cfu/mL in milk (Parker et al., 2018); however, PCR analysis depends on active shedding of *M. bovis* in the milk. This is a challenge because *M. bovis* is shed intermittently (Biddle et al., 2003), and in many cases, milk from infected cows is not necessarily in the BTM at the time of sampling. The result of a single PCR analysis could thus lead to an underestimation of the herd prevalence (Petersen et al., 2016).

One objective of this study was to investigate the prevalence of *M. bovis* in Swedish dairy herds by determining the presence of specific antibodies and *M. bovis* DNA in BTM samples. A second objective was to study herd characteristics and herd location associated with a positive BTM sample using herd health and production data from the DHI database and mapping of the herds.

MATERIALS AND METHODS

This study involved no invasive procedures or handling of animals out of normal routine. Ethical approval or consent to participate was therefore not required.

Study Population and Sampling

Bulk tank milk from 3,144 Swedish dairy herds was collected at the milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden) in November 2019, in conjunction with routine milk quality analysis. Sweden had a total of 3,174 dairy herds at this time, which implies that 99.1% of all dairy herds were included. The samples were collected in 10-mL test tubes containing 1.5 mg of the preservative agent bronopol (2-bromo-2-nitropropane-1,3-diol). The samples were stored at −20°C until analysis.

Laboratory Analysis

All BTM samples (n = 3,144) were analyzed using real-time PCR (PathoProof Mastitis Major 4, Thermo Fisher Scientific), according to the manufacturer's instructions, at Eurofins Steins Laboratory. The cutoff for positive samples was set to cycle threshold <40 according to the manufacturer's instructions.

The samples were sent by postal service to the Swedish University of Agricultural Sciences (**SLU**), Uppsala. Out of all samples, 75 went missing during handling and transportation; these samples were not specific to any region. The remaining 3,069 samples were analyzed for antibodies to *M. bovis* with ID Screen indirect ELISA at the Department of Clinical Sciences, SLU, according to the manufacturer's instructions. The relative amount of antibodies in the samples was calculated as [sample optical density (OD) − negative control OD]/[positive control OD − negative control OD] × 100 (**S/P%**). The BTM samples were analyzed with the overnight incubation protocol and the cutoff for a positive sample

Table 1. Summary of the continuous variables showing the number of herds (n) and the median and interquartile range (IQR) for herds with no antibodies against *Mycoplasma bovis* (negative) and herds with *M. bovis* antibodies (positive) based on a total of 1,583 herds[1]

Item	Negative			Positive			*P*-value[2]
	n	Median	IQR	n	Median	IQR	
Milk production[3]	1,442	10,181	9,151–11,043	97	10,289	9,447–11,326	0.10
BTM SCC[4]	1,485	245	192–303	98	255.5	215–305	0.17
Calving interval,[5] mo	1,461	13.3	12.7–14.1	98	13.2	12.7–14.0	0.83
Age at first calving,[6] d	1,451	841	794–914	97	849.5	793–900	0.69
Cows at >70 d calving to first insemination,[7] %	1,446	21.7	15.0–32.4	97	20.0	14.2–28.1	0.23
Cows at >120 d calving to final insemination,[8] %	1,462	6.6	4.6–8.7	98	7.0	5.3–9.1	0.052
Culling for any reason including cow mortality[9]	1,462	33.6	27.4–40.8	98	36.1	29.9–42.9	0.036

[1]Each herd was tested with a *M. bovis* ELISA on a single bulk tank milk (BTM) sample.

[2]*P*-value from Student's *t*-test.

[3]Mean production per cow (kg of ECM).

[4]Measured as 1,000 cells/mL, arithmetic mean of 12 monthly measurements.

[5]Mean interval between latest calving and the calving before that, for all cows from second lactation giving birth during the 12-mo period.

[6]Mean age at first calving for heifers giving birth during the 12-mo period.

[7]Number of cows in the 12-mo study period with an interval between calving and first insemination of >70 d divided by the mean number of cows with >70 d passed since calving (i.e., including cows calving within 70 d before the study period), not including cows calving within 70 d before the end of the study period.

[8]Number of cows in the 12-mo study period with an interval between calving and final insemination of >120 d divided by the mean number of cows with >120 d passed since calving (i.e., including cows calving within 120 d before the study period), not including cows calving within 120 d before the end of the study period.

[9]Cases per 100 animals at risk.

was set to S/P% $\geq$ 30% as suggested by the manufacturer.

Data Collection

Herd-level data on health variables were retrieved from the DHI database (Växa Sverige) for the period of November 1, 2018, to October 31, 2019 (i.e., the 12-mo period ending just before the BTM sampling). Data on herd size were additionally retrieved from Växa Sverige for the same time period. Of the herds with both PCR and antibody analysis, 2,258 (74%) were affiliated with the DHI program and 3,011 (98%) had data regarding herd size. For the statistical analysis of herd characteristics, only herds from regions with at least 1 positive herd were included. The total number of herds was 2,103, among which 1,583 (75%) were affiliated with the DHI program and 2,059 (98%) had data regarding herd size. Observations were missing for some variables. For each variable, a single value was obtained for each herd, presented in Tables 1 and 2. Data regarding mortality, culling rates, reproductive performance, and veterinary-treated clinical diseases were calculated as cases per 100 animals at risk. Herd size was calculated as the average number of cows (both lactating and dry) over the 12-mo study period. Milk production was calculated as the mean production per cow (ECM, kg) for the 12 mo that data were collected. Bulk tank milk SCC in thousands of cells per milliliter was calculated as the arithmetic mean of 12 monthly measurements. Breed was classified into 4 categories at the herd level, with the main breed consisting of more than 80% of the cows. Distribution of breed at the herd level was Swedish Holstein (**SH**), 31%; Swedish Red (**SR**), 7%; mixed SH and SR, 26%; and other breeds, 36%. The 2 main dairy cow breeds in Sweden are SR and SH.

Statistical Analysis

All variables were checked for outliers and unreasonable values. For the variables "% cows with >70 d between calving and first insemination" and "% cows with veterinary-treated diseases," 28 and 1 observations, respectively, were omitted because of values of >100%. The variables that were not linearly related to the logit of the outcome were either transformed to achieve normal distribution (BTM SCC and "% cows with >70 d from calving to first insemination" were log-transformed; milk production and calving interval were transformed by cubic function), categorized into equally sized groups, or dichotomized by median or by 0 and >0, according to Tables 1 and 2.

The statistical analysis assessed the effects of herd size on herd-level *M. bovis* antibody status (negative/positive) and whether herd-level *M. bovis* antibody status was predictive of the various herd health outcomes.

Table 2. Number of herds in levels of categorical variables, categorized into equally sized groups or dichotomized by median or by 0 and >0, showing herds with no antibodies to *Mycoplasma bovis* (negative) and herds with *M. bovis* antibodies (positive) based on bulk tank milk samples

Variable	Level	Negative (%)	Positive (%)	*P*-value[1]
Herd size (cows)	1: <40	443 (21.6)	9 (6.3)	<0.001
	2: 40–69	573 (28.0)	26 (18.1)	
	3: 70–119	475 (23.2)	38 (26.4)	
	4: >120	557 (27.2)	71 (49.3)	
Calf mortality (0–24 h),[2] %	0: 0–4.99	747 (51.1)	48 (49.0)	0.80
	1: >4.99	714 (48.9)	50 (51.0)	
Early calf mortality (1–60 d)[2,3]	0: 0–1.66	746 (51.1)	34 (34.7)	0.002
	1: >1.66	715 (48.9)	64 (65.3)	
Late calf mortality (2–6 mo)[2,3]	0: 0	988 (67.7)	42 (42.9)	<0.001
	1: >0	472 (32.3)	56 (57.1)	
Young stock mortality (6–15 mo)[2,3]	0: 0	1,021 (70.1)	45 (45.9)	<0.001
	1: >0	435 (29.9)	53 (54.1)	
Culling first parity cows early lactation (0–90 d)[3]	0: 0–1.6	723 (49.5)	29 (29.6)	0.001
	1: >1.6	737 (50.5)	69 (70.4)	
Culling due to udder diseases[3]	0: 0–6.9	732 (50.1)	49 (50.0)	0.99
	1: >6.9	730 (49.9)	49 (50.0)	
Culling due to hoof and leg diseases[3]	0: 0–1.69	742 (50.7)	38 (38.8)	0.023
	1: >1.69	720 (49.3)	60 (61.2)	
Culling due to reproduction diseases[3]	0: 0–6.94	737 (50.4)	43 (43.9)	0.21
	1: >6.94	725 (49.6)	55 (56.1)	
Cow mortality[2,3]	0: 0–4.93	740 (50.6)	41 (41.8)	0.094
	1: >4.93	722 (49.4)	57 (58.2)	
All veterinary-treated diseases[3]	0: 0–16.4	734 (50.2)	53 (54.1)	0.91
	1: >16.4	727 (49.8)	45 (45.9)	
Veterinary-treated clinical mastitis[3]	0: 0–6.7	739 (50.5)	41 (41.8)	0.10
	1: >6.7	723 (49.5)	57 (58.2)	
Veterinary-treated hoof and leg diseases[3]	0: 0	645 (44.1)	29 (29.6)	0.006
	1: >0	817 (55.9)	69 (70.4)	
Heifers >17 mo not inseminated, %	0: 31.95	733 (50.1)	47 (48.0)	0.61
	1: >31.95	729 (49.9)	51 (52.0)	

[1]*P*-value from chi-squared test.
[2]Mortality includes death and euthanization.
[3]Cases per 100 animals at risk.

Each of the herd variables was first evaluated by chi-squared test (χ^2) for categorical variables and Student's *t*-test for the continuous ones. All variables with $P \leq 0.20$ were further analyzed in multivariable logistic or linear regression models, correcting for biologically plausible variables. A backward stepwise approach for model building was used, starting with a full model and at each step eliminating one variable at a time ($P \geq 0.05$) from the regression model to find a reduced model that best explained the data. At each step the variable with the highest *P*-value was removed, and when all remaining variables had a *P*-value ≤ 0.05 the regression model was final. After omitting a variable, previous omitted variables were tested again, and the model was re-examined, and the selection of variable was reviewed again. This was possible because we had a limited number of variables in the full model. The presence of confounding was assessed by examining the effect of each predictor variable on the coefficient of other variables in the model by adding and removing them into and out of the model and examining the change in the coefficients of the remaining model variables. A complete description of the variables included in each regression model is available in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.19323563; Hurri et al., 2022). Model fit was assessed with Hosmer-Lemeshow goodness-of-fit test, and plots of Pearson residuals versus the predicted values were constructed and evaluated for outliers. All statistical analyses were performed using Stata (release 17.0; StataCorp LP).

RESULTS

Prevalence

In total, 147 of the herds (4.8%) tested antibody ELISA positive, and the positive BTM samples ranged from 30.6 to 172.8 S/P%. All the herds tested PCR negative. The true herd-level prevalence of *M. bovis* based on antibodies in BTM was estimated as 3.8% (95% CI 3.0–4.7%), using Epitools Epidemiological Calculators (Sergeant, 2018), based on the diagnostic sensitivity (93.5%) and specificity (98.6%) for the ID Screen antibody ELISA (Andersson et al., 2019). The

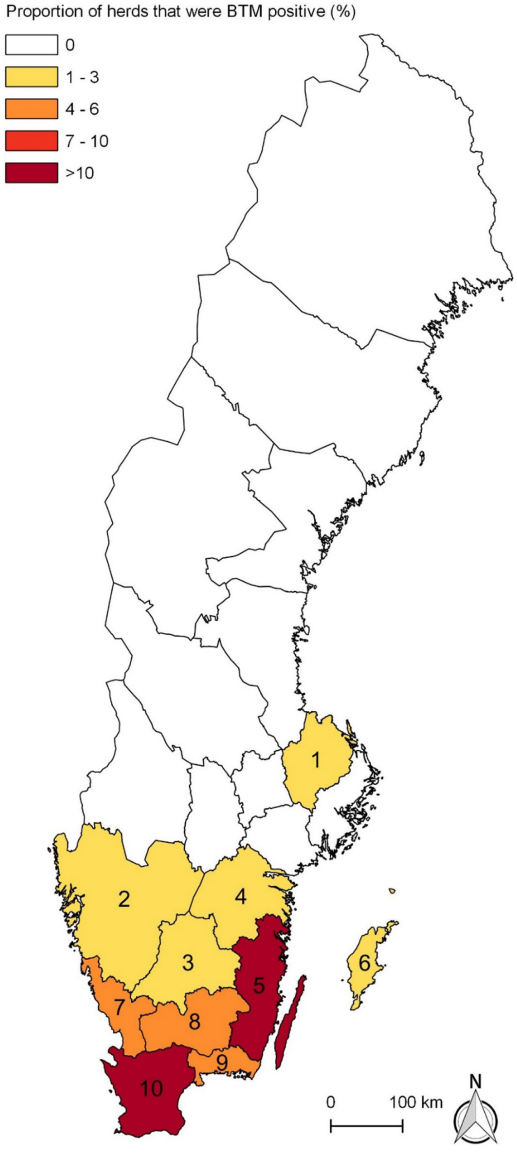

Figure 1. The proportion (%) of herds with antibodies to *Mycoplasma bovis* in bulk tank milk (BTM) for each of the 21 geographic regions in Sweden: 1 = Uppsala (1%, n = 1); 2 = Västra Götaland (3%, n = 16); 3 = Jönköping (3%, n = 10); 4 = Östergötland (3%, n = 7); 5 = Kalmar (13%, n = 41); 6 = Gotland (3%, n = 4); 7 = Halland (4%, n = 6); 8 = Kronoberg (5%, n = 6); 9 = Blekinge (6%, n = 3); 10 = Skåne (20%, n = 53).

apparent herd-level prevalence of *M. bovis* showed regional differences (0–20%), and almost two-thirds of the positive herds (n = 94) were situated in just 2 provinces in the south and southeast of Sweden (Skåne and Kalmar; Figure 1). The rest of the positive herds were located in the other 7 regions in the south, except for 1 positive herd (45.1 S/P%) in Uppsala in central Sweden. The proportion of herds that were BTM positive in each of the 21 regions in Sweden are shown in Figure 1. The term prevalence in this paper refers to herd-level prevalence.

Analysis of Herd Characteristics

Because none of the herds located in the middle and north of Sweden were antibody positive, with the exception of Uppsala, these regions were not included in the analyses of associations between herd characteristics and herd-level seropositivity. The region (isle) of Gotland had 4 positive herds, but these herds were not affiliated with the DHI program. The regions included in the analysis of associations between herd characteristics and herd-level seropositivity were Uppsala, Östergötland, Västra Götaland, Jönköping, Kronoberg, Kalmar, Blekinge, Halland, and Skåne (Figure 1). This corresponds to DHI data from approximately 69% of the *M. bovis* positive herds (98/143) and 76% of the negative ones (1,485/1,960). The DHI herd health variables had between 1,539 and 1,583 observations (Tables 1 and 2), the variation is due to missing values and the exclusion of unreasonable values. For the herd size analysis, Gotland was additionally included and herd size was available from 98% of the positive herds (144/147) and 99% of the negative herds (2,048/2,091) in the regions with at least 1 positive herd.

In the initial screening of association between the herd-level *M. bovis* antibody status (negative or positive) and herd characteristics, 13 out of the 22 variables had a *P*-value of $\leq$0.20, and the variable "culling due to reproduction diseases" had a *P*-value of 0.21 (Tables 1 and 2); these 14 variables were further assessed in the model-building procedure. Each of the 14 variables were tested in linear or logistic regression models correcting for the effect of breed, herd size, milk production, and region. Three variables remained statistically significantly related to antibody status, and 1 variable was borderline statistically significant. The results of these 4 models are presented in Table 3. In summary, larger herds had a higher risk of antibody positivity, and statistically significant associations were found between antibody positivity and having a mortality of more than 0% in older calves (age 2–6 mo) as well as in young stock (age 6–15 mo). Moreover, compared with antibody-negative herds, antibody-positive herds

Table 3. The results of linear and logistic regression models; regression coefficients (Coef.) with SE, *P*-value, odds ratios (OR), and 95% CI of OR, evaluating herd level variables associated with antibody status against *Mycoplasma bovis* measured in bulk tank milk

Outcome	Predictor of interest, level	Coef.	SE	*P*-value	OR (95% CI)	Model corrected for	Model type
M. bovis status (0: negative, 1: positive)	Herd size (cows)					Region	Logistic regression
	1: <40	Referent	0.39	0.12	1		
	2: 40–69	0.61	0.36	0.001	2.00 (0.92–4.37)		
	3: 70–119	1.22	0.35	<0.001	3.77 (1.78–7.97)		
	4: >120	1.54			8.82 (2.35–9.90)		
Calf mortality 2–6 mo (0: 0%; 1: >0%)	*M. bovis* status					Herd size[1]	Logistic regression
	0: negative	Referent			1		
	1: positive	0.60	0.23	0.012	1.83 (1.17–2.86)		
Young stock mortality 6–15 mo (0: 0%, 1: >0%)	*M. bovis* status					Herd size[1]	Logistic regression
	0: negative	Referent			1		
	1: positive	0.57	0.23	0.008	1.77 (1.13–2.77)		
Cows >120 d calving to final insemination[2] (continuous)	*M. bovis* status					Herd size[1] Milk production[1]	Linear regression
	0: negative	Referent					
	1: positive	0.66[3]	0.34	0.052	Not applicable		

[1]Categorized into 4 equal-sized groups.

[2]The percentage of all cows in the herd whose calving to final insemination interval was >120 d.

[3]The proportion of cows that were >120 d from calving to final insemination was 0.66 percentage units higher in herds that were positive for *M. bovis* antibody.

tended to have a higher incidence of cows with more than 120 d between calving and final insemination ($P = 0.052$).

DISCUSSION

In this cross-sectional study we aimed to determine the apparent prevalence of *M. bovis* in Swedish dairy herds using the ID Screen ELISA and to identify herd characteristics associated with a positive antibody test result. Bulk tank milk was sampled from all Swedish dairy herds (3,144). In the analysis of herd characteristics, we used data from the DHI database at Växa Sverige and only herds in regions with positive herds were included (1,583).

This study is the first to analyze antibodies to *M. bovis* in BTM on a national level in Sweden, and to our knowledge, it is the first time the ID Screen has been used in a national screening study. Analyzing BTM antibodies to *M. bovis* is a useful screening tool in the field because it is inexpensive and rapid and shows correlation with the antibody levels in serum of individual cows (Vähänikkilä et al., 2019). Analyzing antibodies in milk with the ID Screen ELISA is also supported by another study, showing good correlation between milk and serum in individual animals (Petersen et al., 2020). In our study, the apparent prevalence of *M. bovis* infection based on antibodies in BTM was 4.8% for the whole country, but with large regional differences ranging from 0 to 20%. The estimated true prevalence for the whole country (3.8%; 95% CI 3.0–4.7%), indicates that Sweden has few *M. bovis*–infected dairy herds. In contrast to PCR, antibody ELISA does not detect circulation of the bacterium but can detect relatively recent previous infections with *M. bovis* in a herd. Antibodies in individual cows have been detected up to 1.5 years after infection (Vähänikkilä et al., 2019), but the duration of antibody responses has not been thoroughly studied. Previous data on BTM antibody prevalence are available from studies in other European countries; for example, the BTM antibody prevalence was 7.1% in Danish herds (Nielsen et al., 2015) and 24.8% in Belgian herds (Gille et al., 2018). In those studies, the BIO K302 ELISA was used. However, the BIO K302 did not have a good correlation between milk and serum (Petersen et al., 2016, 2018). Further, in recent studies the ID Screen has shown a higher sensitivity than the BIO K302 (Andersson et al., 2019; Petersen et al., 2020). Thus, our results clearly show a lower prevalence of *M. bovis* in Swedish dairy herds in comparison with other European countries. A possible explanation for the lower prevalence is the lower cattle density in Sweden than in the other countries, resulting in slower spread of the disease. Further, Sweden was most probably free of *M. bovis* until around 2011, when a few cases were diagnosed in both fattening herds and dairy herds in the most southern region, Skåne (Ericsson Unnerstad et al., 2012). Because our results are based on a single BTM sample from each herd, they may be false-negative results for several reasons; for example, few cows with antibodies may have been present in the herd, antibodies may not have formed yet when the test was done, or infection was only circulating among calves and young stock (Petersen et al., 2016; Parker et al., 2017). As a

consequence, the true herd-level prevalence of *M. bovis* infection in Sweden might be higher than what was found in our study. Nevertheless, the results support for the use of a sensitive antibody ELISA test on BTM to monitor herd exposure. Analyzing repeated BTM samples for antibodies to *M. bovis* may convey a higher security in determining the infection status of herds.

In this study the samples were analyzed with both PCR and antibody ELISA, but no herds were PCR positive on BTM. An earlier national screening in Sweden in 2016 that used PCR analysis on BTM showed an apparent prevalence of 0.3% (n = 10) for *M. bovis* (Landin et al., 2019). The limit of detection for the PCR depends on the gene that is being amplified, and this information was not available for the PCR used in the current study (PathoProof Mastitis Major 4; Thermo Fisher Scientific) (Lönsjö 2020). No information is currently available regarding this PCR test performance against the reference standard of BTM culture (Bauman et al., 2018). The current study showed a low detection frequency using a single PCR on BTM, which might be due to a low within-herd prevalence of *M. bovis* and to the fact that *M. bovis* is shed intermittently in the milk (Petersen et al., 2016). Furthermore, milk from cows going through an active infection with *M. bovis* may not be included in the BTM. In addition, the possibility also exists that *M. bovis* antibodies have developed in response to diseases other than mastitis caused by *M. bovis,* such as respiratory infections or arthritis (Nicholas et al., 2002). Other studies have shown a similar pattern. In Belgium, the percentage of herds testing positive for *M. bovis* was 24.8% based on antibodies in BTM and 7.1% based on PCR analysis of BTM (Gille et al., 2018). In Australian herds, including 19 dairy herds with a history of *M. bovis* disease and 6 herds with no such cases, a much higher percentage of BTM samples were positive by antibody ELISA (39%) than by PCR (4%) (Parker et al., 2017). Altogether, our results support previous findings that PCR testing of BTM highly underestimates the *M. bovis* prevalence.

All herds in the north of Sweden tested negative for *M. bovis* in our study. The positive herds were situated in the south, with the highest apparent prevalence in Skåne (20%) and Kalmar (13%). Although the infection seems to have spread among dairy herds in the south during recent years, Sweden still has a favorable situation regarding *M. bovis* compared with many other countries. Few cases of *M. bovis* mastitis have been reported in Sweden, although underdiagnosis is possible because mastitis samples are not routinely analyzed for *M. bovis* and cows with subclinical mastitis might be culled without bacterial diagnosis. The higher prevalence in the south is probably due to the introduction of *M. bovis* in Sweden, the first cases being diagnosed in Skåne in 2011 (Ericsson Unnerstad et al., 2012). The spread of *M. bovis* in the south could also have been facilitated by the higher cattle density and larger herd size in these regions compared with the northern parts. The herds in the south of Sweden (regions 2–10, Figure 1) have an average of 105 cows per herd compared with the average for the whole country, which is 95 cows per herd. In Skåne the average herd size is 119 cows for herds registered in the DHI database (Swedish Board of Agriculture, 2020; Växa Sverige, 2020). Large herd size is a risk factor for *M. bovis*, probably connected to more introductions of animals and more direct or indirect contacts with other herds (Thomas et al., 1981; Fox et al., 2003). Our study showed a strong association between herd size and a positive BTM sample. Another study identified introduction of animals as a risk factor for the presence of *M. bovis* in a herd (Burnens et al., 1999). We did not have information about the herds' history of introduction of animals, but it is possible that many large herds have expanded relatively recently and therefore are more likely to have introduced cattle from other herds.

In this study we explored the associations between antibody status for *M. bovis* and a set of herd health variables. Two variables were associated with a positive BTM sample: late calf mortality and young stock mortality. *Mycoplasma bovis* infections in calves and young stock commonly present as pneumonia, otitis media, arthritis, or a combination of these disorders (Maunsell and Donovan, 2009). In many cases, the clinical disease becomes chronic and unresponsive to treatment, which leads to increased mortality. Our results show that *M. bovis* status was predictive of mortality in animals that were 2 to 15 mo old, but not those that were younger. A reason could be that pneumonia usually affects calves more than 2 wk of age. These animals might be treated several times, and therefore, chronic effects and mortality are seen in older animals (2–15 mo). This finding is also supported in a study by Petersen et al. (2019) in which undesired early departure of heifers >90 d of age was more common in *M. bovis* antibody-positive herds than in negative herds. In that study, however, early departure included premature culling of heifers, while we only had information on mortality (death and euthanization).

The current study has some limitations, aside from the diagnostics being done on BTM, as discussed above. We did not have information on many herd-related factors, and thus we could not control for them. In addition, we had no information on when *M. bovis* was introduced in the seropositive herds, the severity of the disease, and how it had developed over time in each herd before sampling.

Further research in *M. bovis*-infected herds is needed to determine the effect on herd health and mortality. Also, the increased risk for *M. bovis* antibody positivity as herd size increases, could imply a different contact pattern to other herds compared with that of smaller herds, which calls for further research. The possibility of reverse causality contributing to this association seems unlikely as farmers in Sweden are not able to increase the number of milking cows to compensate for some cows being in poor health because indoor housing systems limit the herd size. The study also showed a trend for a positive BTM sample being associated with an increasing percentage of cows with more than 120 d between calving and final insemination. This result could be a sign of increased subclinical disease due to *M. bovis* that affects fertility (Fox, 2012), but it could also be due to other herd factors. The effect on fertility would be interesting to investigate further in *M. bovis*–positive herds.

In Sweden, the most severe *M. bovis*–related disease has been seen in fattening herds, although calves in dairy herds also appear to suffer from *M. bovis* (Ericsson Unnerstad et al., 2012; Hurri et al., 2021). Substantial economic losses are connected to mortality of calves and young stock in dairy herds, and calculations show that the cost is 315 euro per case (heifers) for late calf mortality and 680 euro per case for young stock mortality in 2020 (Växa Sverige, animal welfare costs online tool, https://www.vxa.se). To reduce the costs of disease and secure animal welfare, the whole cattle sector in Sweden has now decided to come together to prevent *M. bovis* spread (Växa Sverige, 2021).

CONCLUSIONS

In this cross-sectional study, we found a higher prevalence of *M. bovis* in Sweden than what was previously known. The herds with an antibody-positive BTM sample were all situated in the south of Sweden, which correlates well with earlier studies and reports from the field. Analyzing BTM by PCR seems unsuitable, owing to the low detection frequency in this study. Analyzing antibodies can provide a more correct prevalence of *M. bovis* infection and be a useful tool to identify infected herds. Large herd size was identified as a risk factor for infection. The association between infection status, as measured by BTM antibody ELISA, and young stock and late calf mortality suggests that *M. bovis* infection affects animal health and welfare in Swedish dairy herds. The relatively low prevalence of *M. bovis* in Sweden is a strong motivation to minimize the spread of this disease and reduce the costs for farmers and the consequences on animal health and welfare.

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A longitudinal study of the dynamics of *Mycoplasma bovis* antibody status in primiparous cows and bulk tank milk in Swedish dairy herds

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ABSTRACT

Mycoplasma bovis is an important pathogen causing pneumonia, mastitis, and arthritis in cattle all over the world entailing reduced animal welfare and economic losses. In this longitudinal study, we investigated the presence of *M. bovis* antibodies in bulk tank milk (BTM) and in milk from primiparous (PP) cows at 4 sampling occasions over 2 yr. Herd characteristics associated with a positive antibody test result in PP cows were investigated. The participating dairy herds (n = 149) were situated in southern Sweden, samples were collected and analyzed with ID Screen antibody ELISA. Information on herd characteristics was retrieved from the national DHI database. To identify herd characteristics associated with the presence of antibodies in PP cows, mixed linear regression with herd and sample as random factors were used. The apparent herd-level prevalence of *M. bovis* infection based on antibodies in BTM was 17%, but with the addition of PP cows, the prevalence increased to 28%. The results showed that larger herds and introduction of cattle was associated with higher antibody levels in PP cows. In conclusion, this study showed a clear difference in the apparent prevalence of *M. bovis* infection based on antibodies in BTM or in PP cows: The number of positive herds was almost doubled when including PP cows. This motivates repeated sampling of a few PP cows to find newly infected herds in an early stage. Finally, the results showed that introduction of cattle influences the level of *M. bovis* antibodies. This is important in the control and prevention of further spread of the infection. It is essential for free herds to know their *M. bovis* status, and antibody testing is highly recommended if introducing cattle.

Key words: heifers, ELISA, *Mycoplasmopsis bovis*, external biosecurity

INTRODUCTION

Mycoplasma bovis causes disease in cattle, with pneumonia, arthritis, and mastitis being the most common clinical presentations (Nicholas and Ayling, 2003; Maunsell et al., 2011). These infections often become chronic and respond poorly to antibiotic treatment, leading to economic losses for the farmers and reduced animal welfare (Maunsell et al., 2011). *Mycoplasma bovis* was first diagnosed in the United States in 1961 (Hale et al., 1962) and has since been detected in cattle all over the world (Nicholas and Ayling, 2003). During the last decade, the importance of *M. bovis* has escalated due to introduction in countries that were previously free, outbreaks in major dairy-producing countries, and the report of a new strain spreading in the Northern European countries (Pothmann et al., 2015; Haapala et al., 2018; Tardy et al., 2020). The most recent introductions were reported in Sweden in 2011, in Finland in 2012 and in New Zealand in 2017 (Dudek et al., 2020; Jordan et al., 2021; Hurri et al., 2022). *Mycoplasma bovis* can cause serious illness, and it may lead to increased use of antimicrobial drugs and increased risk of resistance to several antimicrobial drugs (Gautier-Bouchardon et al., 2014; Klein et al., 2019). There have been numerous efforts to develop efficacious vaccines against *M. bovis* but without success (Perez-Casal et al., 2017). Therefore, there is a need to control and prevent the infection from spreading to new herds and to be able to detect infected cattle, especially subclinically infected animals (Caswell and Archambault, 2007).

Diagnosing *M. bovis* on cattle has historically been done by culture, and later PCR, on milk or nasal swabs, but due to the intermittent shedding of *M. bovis*, serology could be a better option or a supplementary method (Park-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

er et al., 2017). Asymptomatic carriers are a diagnostic challenge, and analyzing antibodies could be a method to identify such animals (Maunsell et al., 2011). Evaluation of commercial *M. bovis* antibody ELISA has been done in recent studies using Bayesian latent class analysis. In these studies, the IDvet ELISA (ID Screen *Mycoplasma bovis* Indirect, Grabels, France) showed a high sensitivity (92.5%–94%) and specificity (92%–99.3%; Andersson et al., 2019; Veldhuis et al., 2023; McAloon et al., 2024). Several studies have been investigating the antibody dynamics and course of *M. bovis* infection in dairy herds using ELISA, to detect antibodies (Vähänikkilä et al., 2019; McCarthy et al., 2021; Penterman et al., 2022). To identify herds that have been exposed to *M. bovis*, herd-level screening is needed; antibodies in bulk tank milk (**BTM**) or milk from individual animals could be a useful tool for this purpose (Petersen et al., 2016). The duration of antibodies is not well understood. In a Finnish study, some animals remained seropositive to *M. bovis* for at least 1.5 yr after the index case, regardless of clinical symptoms of *M. bovis* infection being present on the farm (Vähänikkilä et al., 2019). The antibody response in individual cows was dynamic and varied a lot between cows in a Danish study (Petersen et al., 2018).

There is a need for increased knowledge about *M. bovis* prevalence and optimal diagnostic strategy in dairy herds to effectively prevent and control the disease. Testing a small sample of primiparous (**PP**) cows has been found useful in monitoring herd infection status for other infections, such as bovine respiratory syncytial virus and bovine coronavirus (Ohlson et al., 2013). In Sweden, the prevalence of *M. bovis* at herd level was 4.8% in a national screening of antibodies in BTM performed in 2019, with large regional differences ranging from 0 to 20%, with a higher prevalence in the south of Sweden (3%–20%; Hurri et al., 2022). Since the first cases in 2011, *M. bovis* has spread in dairy herds and fattening herds in the south of Sweden, symptoms being primarily pneumonia and arthritis in feedlot calves, but very few cases of mastitis in dairy cows (Hurri et al., 2021).

Recent studies have identified purchase of cattle (Schibrowski et al., 2018; Murai and Higuchi, 2019; Fujimoto et al., 2020) and the use of a breeding bull (Gille et al., 2018) as risk factors for introduction of *M. bovis* into dairy herds. Other studies have also concluded that large herd size is a risk factor for *M. bovis* infection (Thomas et al., 1981; Haapala et al., 2021; Hurri et al., 2022). The costs of introducing *M. bovis* disease in the herd include production losses, veterinary costs, labor for treatment and care of sick animals, mortality, premature culling, and costs for implementing diagnostic and control measures (Maunsell et al., 2011). Dairy cows infected with *M. bovis* showed higher SCC, produced less milk, and had lower milk fat and urea content compared with *M. bovis*-negative dairy cows in a study from Estonia (Timonen et al., 2017). However, BTM SCC were not higher in *M. bovis*-positive herds compared with negative herds in a study in United States (Fox et al., 2003). The effects and costs associated with calf respiratory disease include reduced fertility, increased age at first calving, and reduced milk production later in life (Maunsell and Donovan, 2009). A recent cross-sectional study by Hurri et al. (2022) showed that there was a higher late calf (2–6 mo of age) and young stock (6–15 mo of age) mortality in herds with seropositive BTM. Effects on animal health and performance need to be further investigated by following dairy herds over time.

In this study, the aims were (1) to monitor the changes in *M. bovis* antibody status in BTM and milk from PP cows in Swedish dairy herds over time, and (2) to investigate potential risk factors and herd health variables associated with *M. bovis* antibodies in primiparous cows.

MATERIALS AND METHODS

Study Population

Five regions (Halland, Kalmar, Skåne, Västra Götaland, and Östergötland) in the south of Sweden with previously known cases of *M. bovis* were selected for the study. Dairy herds in these regions with an average of more than 70 cows (both lactating and dry) on a yearly basis and affiliated with the DHI program (Växa Sverige) were invited to participate in the study. A list of herds (n = 976) fulfilling these selection criteria was retrieved from the dairy farmers' association, Växa Sverige, and all were invited by mail. Out of these, 139 herds (14%) agreed to participate in the study. Another 10 herds joined later after contact with participating herds or due to *M. bovis* being detected on the farm. There were 4 herds that stopped delivering milk during the study and therefore dropped out.

The DHI database collects and stores cow data from monthly milk testing, disease treatments, and production parameters. Around 70% of the dairy herds in Sweden comprising 77% of the cows were affiliated eith the DHI program year September 1, 2019 to August 31, 2020 (Cattle Statistics 2021, Växa Sverige, 2021). In our study, 87% of the herds (n = 130) were available in the DHI database; the other herds had withdrawn from the DHI program after the start of the study.

Sampling Procedures

Herds were sampled 4 times at ~6-mo intervals, in (1) September 2019, (2) February 2020, (3) November 2020, and (4) May 2021, with a 2-mo long sampling window each time (Figure 1). For each herd, on each sampling

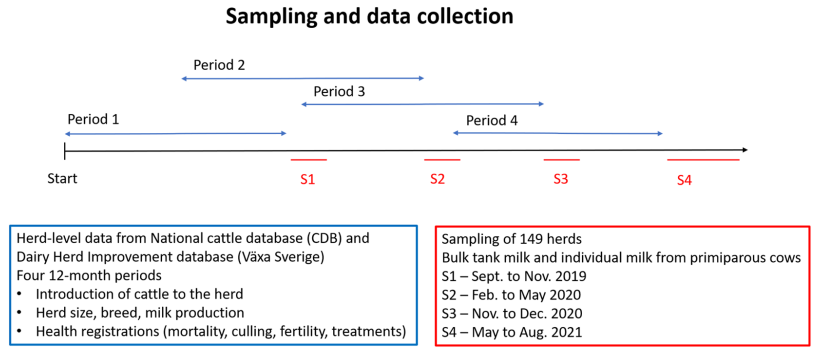

Figure 1. Longitudinal study with sampling of 149 herds 4 times (S1–S4) with approximately 6 mo intervals for 2 yr. Herd-level data retrieved in 12-mo periods (period 1–4) before the start of the sampling.

occasion, milk samples were collected from the 3 youngest home-bred PP cows and BTM. The samples were collected at the milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden), in conjunction with the routine milk quality analysis. Between 0 and 9 PP cows were sampled from each herd, depending on available PP cows at the monthly test milking. To be included in the primiparous sampling, the cows had to be within 6 mo after calving. The samples were collected in 10-mL test tubes containing 1.5 mg of the preservative agent Bronopol (2-bromo-2-nitropropane-1,3-diol) and sent by postal service in ambient temperature to the Swedish University of Agricultural Sciences (**SLU**), Uppsala, Sweden. The samples were stored at −20°C until analysis.

There were 19 herds not affiliated with the DHI database, and from these herds, samples from PP cows could not be collected automatically from the milk testing laboratory. In addition, in 19 herds no PP cows were test milked during the sampling window. Therefore, sample kits were sent out to these farmers to collect and submit milk samples from PP cows. These samples were received at the laboratory between February 2020 and August 2021. Not all herds submitted samples, and in total, 10% to 20% (n = 14–31) of the herds lacked PP samples at one or more sampling occasions.

Milk Analysis

The samples were analyzed for IgG antibodies to *M. bovis* with ID screen indirect ELISA, (IDvet, Grabels, France) at the Department of Clinical Sciences, SLU, in accordance with the manufacturer's instructions. The relative amount of antibodies in the samples was calculated as [sample optical density (OD) − negative control OD]/(positive control OD − negative control OD) × 100 (S/P%). The milk samples were analyzed with the overnight incubation protocol and the cut-off S/P% ≥30% was applied.

Assessment of Infection Dynamics on Herd Level

Herd-level *M. bovis* antibody status was defined as 1 of the 6 following categories at each sampling occasion: (1) BTM and milk from PP cows negative, (2) BTM negative and PP missing, (3) BTM negative and PP positive, (4) BTM and PP positive, (5) BTM positive and PP missing or (6) BTM positive and PP negative. If at least one PP cow was positive, the herd was categorized as PP positive.

Bulk tank milk antibody level was compared with the number of positive PP cows (0, 1, 2, or 3), for all sampling occasions. Herds with more than 3 sampled PP cows (n = 199) were transferred to one of the other categories by dividing the number of positive PP cows with the total number of PP cows. Herds with less than 3 PP cows sampled (n = 13) were included in group 0, 1. or 2. Student's *t*-test was used to evaluate the correlation between the number of antibody-positive PP cows and the antibody level (S/P%) in BTM.

Data

Herd-level data on health and production variables were retrieved from the DHI database. The data were aggregated in four 12-mo periods calculated backward from the start of each sampling period: (1) September 1, 2019, (2) February 1, 2020, (3) November 1, 2020, and (4) May 1, 2021 (Figure 1). Data regarding mortality, culling rates, reproductive performance, and veterinary-reported clinical diseases were calculated as

cases per 100 animals at risk. Herd size was calculated as the average number of cows (both lactating and dry) over the 12 mo. Milk production was calculated as the mean production per cow in kilograms of ECM for the 12-mo period. Bulk tank milk SCC in thousands of cells/mL was calculated as the arithmetic mean of 12 monthly measurements. Breed was classified into 4 categories on herd level, the main breed constituting more than 80% of the cows. Distribution of breed was Swedish Holstein (**SH**) 45% of the herds, Swedish Red (**SR**) 15%, mixed SH and SR 28% and other breeds 12%. SR and SH are the 2 main dairy cow breeds in Sweden. Herd-level data in 12-mo periods regarding herd size (i.e., number of female animals >24 mo old), number of introduced cattle, and number of herds that the introduced cattle originated from (**INHERDS**) were collected at the Swedish Board of Agriculture covering the period from 2018-09-01 to 2021-08-31 (Swedish Board of Agriculture, https://jordbruksverket.se/). Introduced cattle includes all cattle purchased to the farm and the farm's own cattle returning from another external farm or from pasture, located more than 500 m away from the main farm. The data from the National Cattle Database were aggregated in 12-mo periods before the exact date of sampling of the PP cows, but if the sampling date was missing, the period 12 mo before the start of each sampling period was used. Data on herd size (number of milking cows) in 15 herds not affiliated with the DHI were retrieved from a questionnaire answered by these farmers during the fall 2020. In Figure 1, the data collection and sampling periods are visualized.

Statistical Analysis of Risk Factors

The risk factor analysis assessed the effects of herd size and introduction of cattle on *M. bovis* antibody status in PP cows. The analysis also included predictors such as region, breed, INHERDS, median age of the introduced cattle, and antibody level in BTM. We used individual antibody ELISA test results from PP milk samples, (S/P%), as the continuous outcome variable in the model. The outcome variable was log-transformed to achieve normal distribution of the residuals. Each of the herd-level variables was first evaluated univariably in a linear mixed regression model with herd and sample as random effects. A threshold of $P \leq 0.20$ was chosen for detecting potential risk factors to be included into the multivariable models. A manual backward stepwise elimination was used to exclude nonsignificant ($P > 0.05$) variables from the multivariable model, to find the reduced model that best explained the data. At each step the variable with the highest P-value was removed and when all remaining variables had a P-value < 0.05 the regression model was final. After omitting a variable, previously omitted variables were tested again, and the model was re-examined. This was possible because we had a limited number of variables in the full model. Model evaluation was assessed with normal probability plots, and plots of residuals versus the predicted values were constructed and evaluated for outliers.

Statistical Analysis of Health and Production Variables

The statistical analysis assessed if the *M. bovis* antibody status at herd level was predictive of the various herd-level production and health variables. For the *M. bovis* status, 3 categories were possible: (1) negative in both BTM and PP cows, (2) negative BTM and positive PP cows, or (3) positive BTM and positive, negative, or missing PP cows. Each of the herd variables was first evaluated univariably in a multilevel mixed-effects negative binomial regression with herd and sample as random effects. Variables with $P \leq 0.20$ were further analyzed in multivariable multilevel mixed-effects negative binomial regression model for each variable, correcting for biologically plausible variables such as herd size, introduction of cattle (yes or no), milk production, and breed, when adequate. A complete description of the variables offered to each regression model is available in Supplemental Table S1 (see Notes). All statistical analyses were performed using Stata (release 15.1; StataCorp LP, College Station, TX).

RESULTS

Antibody Prevalence at Herd Level

At start, there were 139 farms participating in the study, but due to dropouts (n = 4) and new farms (n = 10) added in the study period, there was a maximum of 145 sampled farms at a single sampling occasion (Table 1). The median herd size was 150 cows (interquartile range [**IQR**] = 87–247).

Geographical Distribution of Herds

The distribution of participating herds and their *M. bovis* antibody status in BTM and milk from PP cows, including changes in status, is presented in Figure 2. Change in status was defined from sampling 1, herds negative at sampling 1 and positive in either sampling 2, 3, or 4, were considered new positive herds. All the herds (n = 10) that joined the study after sampling 1 had the same status the whole period, 4 were negative and 6 were positive both in BTM and PP cows. One-third of the participating herds were situated in Västra Götaland and this region also had the highest number of new positive

Table 1. Antibodies to *M. bovis* in samples collected from bulk tank milk (BTM) and from primiparous (PP) cows on herd-level[1]

Sampling occasion	Herds with BTM samples, n	Herds with positive BTM, n (%)	Herds with BTM and PP samples, n	Herds with positive PP, n (%)	Herds with positive PP and negative BTM, n	Herds with negative PP and positive BTM, n
1	139	22 (15.8)	108	31 (28.7)	15	1
2	144	24 (16.6)	130	31 (23.8)	11	4
3	145	25 (17.2)	126	40 (31.7)	16	1
4	145	26 (17.9)	123	36 (29.3)	12	1

[1]PP positive herds had at least one antibody-positive PP cow.

herds. The highest number of BTM positive herds were situated in the southeast of Skåne.

Presence of M. bovis Antibodies on the Farms

In total, 18% (450/2,448) of the PP samples were positive, and the individual values for the positive cows varied between 30.2 and 354.5 S/P%. For the BTM, 17% (98/575) of the samples were positive, and the different values for the positive samples varied between 30.8 and 156.4 S/P%. The number of antibody-positive PP cows in each herd was significantly correlated with the antibody level (S/P%) in BTM ($P < 0.001$, Student's t-test, Figure 3). For all 4 sampling occasions, there were in total 487 samplings with test results from both BTM and PP cows.

The results for the analysis of BTM and PP samples at each sampling occasion are shown in Figure 4. At the study commencement 76 herds out of 139 were negative in both BTM and PP samples, 15 herds were negative in BTM and positive in PP sample, and 16 were positive in both sample types, 31 herds had only BTM samples (26 negative and 5 positive). Considering a herd positive at a sampling occasion either on positive BTM or at least

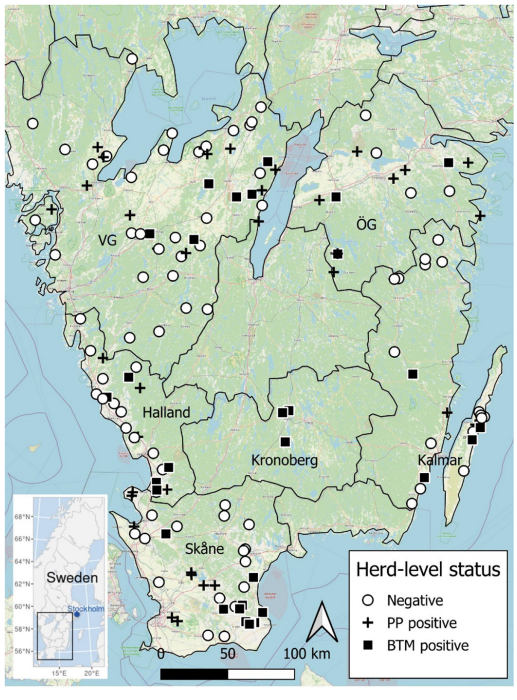

Figure 2. Geographical distribution of the participating herds, stratified by antibody status to *Mycoplasma bovis* in bulk tank milk (BTM) and milk from primiparous (PP) cows sampled 4 times between September 2019 to August 2021. Negative = negative BTM and negative PP milk, PP positive = negative BTM and positive PP milk, BTM positive = positive BTM and positive or negative or missing PP milk. VG = Västra Götaland, ÖG = Östergötland.

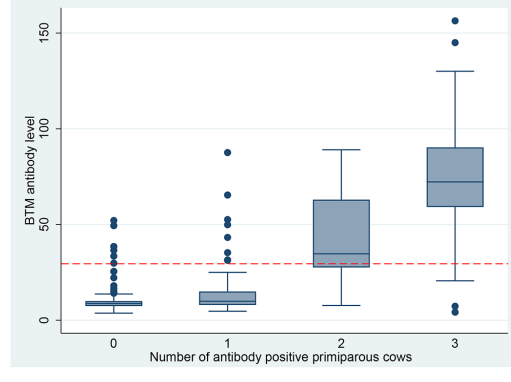

Figure 3. Bulk tank milk antibody level compared with the number of positive primiparous (PP) cows at all sampling occasions. The red dashed line represents the cut-off for the ELISA (S/P ≥ 30% = antibody positive). Herds with more than 3 sampled PP cows (n = 199) were transferred to one of the other categories by dividing the number of positive PP cows with the total number of PP cows sampled. Herds with less than 3 PP cows sampled (n = 13) were included in group 0, 1, or 2. The box is drawn from lower quartile (Q1) to the upper quartile (Q3) with a horizontal line drawn inside it to denote the median. The boundaries of the whiskers is based on the 1.5 IQR value from above Q3 and below Q1. The dots represents outliers outside the above range.

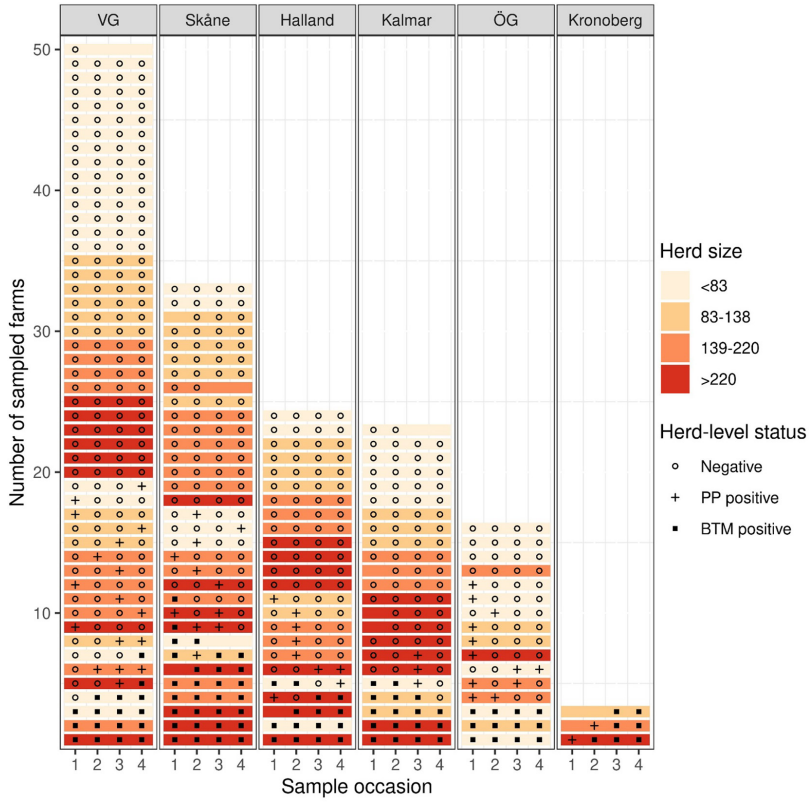

Figure 4. Herd-level results from analysis of antibodies to *Mycoplasma bovis* in bulk tank milk (BTM) and in individual milk from primiparous (PP) cows. Milk was sampled on 4 occasions: (1) autumn 2019, (2) spring 2020, (3) autumn 2020, and (4) spring/summer 2021. The symbols in the graph show the herd status at each sampling and the colors show the herd size. The herds are divided into columns by region, Västra Götaland (VG), Skåne, Halland, Kalmar, Östergötland (ÖG), and Kronoberg. Each horizontal line represents the time series for a single herd. If there is no symbol in the colored field, samples are missing.

1 PP cow positive, changes from negative to positive (on the next sampling) occurred 29 times (4 times in BTM and 25 times in PP). Changes from positive to negative at the next sampling occurred 34 times (5 times in BTM and 29 times in PP). Eleven herds went both from positive to negative and from negative to positive during the period of sampling. Most of the herds (n = 104, 70%) had the same status on all sampling occasions (22 positive and 82 negative). There were 67 herds with at least 1 positive sample. Out of these, all herds with negative BTM (n = 35) had both positive and negative PP samples. Most of the herds with positive BTM had also all PP samples positive (n = 24/32). Antibodies in either BTM or PP cows were detected in 63% (24/38) of the herds with more than 220 cows, 44% (16/36) in herds with 139–220 cows, 38% (14/37) in herds with 83 to 138 cows and 34% (13/38) in herds with less than 83 cows.

Risk Factors

In the initial screening of associations between *M. bovis* antibody status in PP cows and herd-level risk factors, 5 out of the 7 variables had a *P*-value of ≤0.20 and were further assessed in the model building procedure (Table 2). The variables region and breed had *P*-values above 0.20. In the final model, 3 variables remained significantly related to antibody status (Table 3). There were higher antibody levels in PP cows in larger herds, in

Table 2. Summary of the continuous variables showing number of individual milk samples from primiparous cows (n), median and interquartile range (IQR), for samples with no antibodies to *Mycoplasma bovis* (negative), and samples with such antibodies (positive) based on, in total, 2,448 samples from 143 herds at 4 different sampling occasions between September 2019 and August 2021[1]

	Negative			Positive			
Item	n	Median	IQR	n	Median	IQR	*P*-value[2]
Primiparous cow antibody level, S/P%	1,998	7.8	5.2–11.6	450	92.0	58.2–139.4	NA
BTM antibody level	1,997	8.7	7.5–10.2	441	66.4	49.9–82.1	<0.001
Herd size, cows	1,998	141	86–221	450	235	124–511	<0.001
No. of ingoing cattle	1,998	1	0–36	450	18	0–142	<0.001
Age of ingoing cattle, d	1,091	677	453–785	337	667	453–761	0.156
INHERDS[3]	1,998	1	0–2	450	2	0–4	0.088
Milk production[4]	1,866	11,060	10,359–11,740	383	11,321	10,476–12,129	0.886
BTM SCC[5]	1,865	244	196–288	393	241	193–273	0.974
Calf mortality 0–24 h[6] (%)	1,865	5.5	3.8–7.2	393	4.8	3.4–7.1	0.792
Early calf mortality (1–60 d)[6,7]	1,865	3	1–5	393	3	2–5	0.309
Late calf mortality (2–6 mo)[6,7]	1,865	1	0–2	393	1	0–2	0.142
Young stock mortality (6–15 mo)[6,7]	1,865	1	0–2	393	1	0–2	0.397
Cow mortality[6,7]	1,865	5	4–7	393	6	4–9	0.167
Culling of first parity cows in early (0–90 d) lactation[7]	1,865	3	1–6	393	4	2–7	0.310
Culling due to udder diseases[7]	1,865	8	5–11	393	8	5–11	0.930
Culling due to hoof and leg diseases[7]	1,865	3	2–5	393	4	2–5	0.202
Culling due to reproduction disorders[7]	1,865	8	5–10	393	8	5–11	0.178
Culling due to any reason including cow mortality[7]	1,865	35	31–41	393	38	34–42	0.143
All veterinary-treated diseases[7]	1,865	23	15–31	393	21	10–32	0.203
Veterinary-treated clinical mastitis[7]	1,865	10	6–14	393	8	4–10	0.223
Veterinary-treated hoof and leg diseases[7]	1,865	2	1–4	393	2	0–4	0.292
Calving interval,[8] mo	1,865	13	12–13	393	13	13–13	0.871
Age at first calving,[9] d	1,865	795	761–838	393	807	758–848	0.754
Heifers >17 mo not inseminated, %	1,865	15	6–27	393	17	7–34	0.463
Cows >70 d calving to first insemination,[10] %	1,865	17	11–24	393	16	13–20	0.953
Cows >120 d calving to final insemination,[11] %	1,865	6	5–8	393	7	5–8	0.479
Breed							
Swedish Red	278			202			Referent
Swedish Holstein	793			39			0.099
Mixed Red/Holstein	650			135			0.121
Other	173			20			0.604
Region							
Halland	373			48			Referent
Kalmar	295			47			0.941
Kronoberg	3			51			<0.001
Skåne	315			131			0.225
Västra Götaland	741			106			0.754
Östergötland	271			67			0.420

[1]Herd and sampling occasion were used as random effects. All variables have been calculated in 12-mo periods preceding sampling.

[2]*P*-value from univariable linear multilevel mixed regression with *M. bovis* antibody status in primiparous cows as outcome in the risk factor model. *P*-value from multilevel mixed-effects negative binomial models with health and production characteristics as outcome in the effect model.

[3]Number of herds where introduced cattle originate from in 12-mo period preceding sampling.

[4]Mean production per cow per 12 mo preceding sampling in kilograms of ECM.

[5]Bulk tank milk somatic cell count in 1,000 cells/mL, arithmetic mean of 12 monthly measurements.

[6]Mortality includes death and euthanasia per 12 mo preceding sampling.

[7]Cases per 100 animals at risk for 12 mo preceding sampling.

[8]Mean interval between latest calving and the calving before that, for all cows from second lactation giving birth during the 12-mo period preceding sampling.

[9]Mean age at first calving for heifers giving birth during the 12 mo preceding sampling.

[10]Number of cows, in the 12 mo preceding sampling, with an interval between calving and first insemination of >70 d divided by the mean number of cows with >70 d passed since calving (i.e., including cows calving within 70 d before the study period, not including cows calving within 70 d before the end of the study period).

[11]Number of cows, in the 12 mo preceding sampling, with an interval between calving and final insemination of >120 d divided by the mean number of cows with >120 d passed since calving (i.e., including cows calving within 120 d before the study period, not including cows calving within 120 d before the end of the study period).

herds that had introduced a higher number of cattle, and in herds with higher antibody levels in BTM. There were 2,248 observations from 143 herds and 4 different sampling occasions included in the analysis. The median age of the introduced animals was 674 d (IQR = 453–770). This variable was not included in the final model because

Table 3. Mixed linear regression model with herd and sampling occasion as random effects, regression coefficients with SE, and *P*-values evaluating herd-level variables associated with antibody status to *M. bovis* measured in milk from primiparous cows

Outcome	Predictor of interest	Coefficient	(SE)	*P*-value
M. bovis status primiparous cows	Herd size (cows)	0.001	(0.000)	0.003
	Introduction of cattle to the herd	0.001	(0.000)	0.006
(log-transformed)	BTM antibody level	0.025	(0.001)	<0.001

half of the herds had no introductions of cattle. When age was introduced in a similar model only including the herds with introductions of cattle, age of the introduced cattle was not a significant risk factor.

Effects on Health and Production

After the first univariable analysis there were 4 out of 20 variables (late calf mortality [2–6 mo], cow mortality, culling due to reproduction disorders, and culling due to any reason) with a *P*-value of ≤0.20 in the regression models (Table 2). These variables were further assessed in the model building procedure. Each of the 4 variables were tested in mixed negative binomial regression models correcting for the effect of breed, herd size, milk production, region, and introduction of cattle (yes or no). After evaluation of the models, there were no variables that remained significantly ($P \leq 0.05$) related to the herd-level antibody status in BTM and PP cows.

DISCUSSION

In the current study, sampling of BTM and milk from PP cows was repeated 4 times in 149 herds over 2 yr. The samples were analyzed for *M. bovis* antibodies with ID Screen ELISA. Together with *M. bovis* antibody status in the herds, data on introduction of cattle, herd size, health, and production in the herds were analyzed.

In this study, including individual samples from a few PP cows in addition to analyzing BTM alone increased the number of detected positive herds by 50% to 100%. The finding of positive PP cows and negative BTM suggests that few cows in the milking herd are seropositive. This could either be a result of a low within-herd transmission of *M. bovis* infection, or a recent introduction in the young cows. The risk of false positive test results contributing to this finding is low, around 1%, considering the high specificity (98.6%) of the ELISA (Andersson et al., 2019) and the prevalence of 18% detected among PP cows. In BTM positive herds, at least 30% of the lactating cows had antibodies in a study in Denmark (Petersen et al., 2016). In the few herds that showed positive BTM and negative PP cows, the sampling strategy failed to detect the antibody-positive cows, possibly older cows. Good biosecurity routines may have prevented the infection of young cows or *M. bovis* was no longer circulating in the herd, while historic antibodies were measured in BTM. In most herds with positive BTM, the PP samples were positive throughout the study period. In herds with negative BTM and positive PP cows, there was more variation in the PP results between samplings, probably due to a low within-herd seroprevalence and different cows sampled on each occasion (Petersen et al., 2016; Penterman et al., 2022). The BTM antibody levels in most of the positive herds did not decrease over a period of 2 yr. These findings support previous studies that BTM could be a good herd-level screening tool (Parker et al., 2017; Petersen et al., 2018; Salgadu et al., 2022).

Antibodies remain detectable in the herd after symptoms of clinical *M. bovis* disease have waned, but asymptomatic carrier animals may prevail, making the herd infection status difficult to assess (Maunsell et al., 2011; Penterman et al., 2022). Considering this, finding antibody-positive herds early is important in the work to prevent the spread of *M. bovis*. Antibody levels in PP cows have not been examined for *M. bovis* in other studies. The strategy to sample young cows was based on the assumption that they would reflect an active transmission of the infection in the herd better than older cows, since the duration of the antibody response (in the latter) may be quite long (Vähänikkilä et al., 2019). In previously *M. bovis*-free herds, PP cows might be the group first infected by *M. bovis* because they may have more contact with other herds, for example through external contractors rearing heifers from more than one dairy herd or by purchased animals being introduced into this age group. In this study, the median age of introduced animals was 674 d, approximately 22.7 mo, which seems to comply with the knowledge that pregnant heifers are the animals most often introduced to Swedish dairy herds. Therefore, we assumed that detecting antibody-positive PP cows would be a way to detect recent introduction of infection.

Introduction of cattle was correlated with increased antibody levels in PP cows. The introduction of cattle posed a significant risk factor, even though it also involved the movement of the farm's own cattle, which did not come into contact with cattle from other herds. It is well known that asymptomatic carrier animals play a big role in transmission of *M. bovis* (Maunsell et al., 2011). It has been shown that introduction of purchased animals

is a risk of getting *M. bovis* (Burnens et al., 1999) and this risk is decreased if herds only buy animals from controlled herds and only have other contacts (shared pasture, animal exhibitions) with *M. bovis* antibody negative herds (Dudek et al., 2020). There might, however, be a possibility that the infection is spreading to new herds through other pathways. The extent of transmission by fomites, clothes, humans, and semen is not fully understood, but these are possible risks of disease introduction (Haapala et al., 2018; Schibrowski et al., 2018). In the present study, larger herd size was a significant risk factor for having *M. bovis* antibodies. This was also previously shown in a study based on BTM samples from a national screening (Hurri et al., 2022) and in a study by Thomas et al. (1981). Larger herds and expanding herds have more contacts with other herds, both directly and indirectly, which entails a risk of introducing new pathogens (Fox et al., 2003; McAloon et al., 2022). The herds in our study had a median herd size of 150 cows (IQR = 87–247), which is higher than the mean herd size of 94 milking cows in Sweden (Swedish Board of Agriculture, 2019). This could have affected our results with a higher percentage of positive herds in the regions included in the present study (southern Sweden) compared with the national screening in 2019. Regions in the southern parts of Sweden were targeted in this study to find positive herds, making the results more comparable to other countries where *M. bovis* is endemic (Maunsell et al., 2011; Dudek et al., 2020). In this study about 17% of the herds were BTM antibody positive, compared with the national screening in 2019 where 8% (range 3%–20%) were positive in these regions (Hurri et al., 2022). This finding might reflect an increased prevalence over time, but it is also possible that farms with *M. bovis* infection were more prone to participate in the present study. However, the access to positive herds was beneficial for the study, because we wanted to investigate and monitor *M. bovis* over time. Therefore, we believe that the number of herds was satisfactory even though the participation rate was 14%, reflecting that willingness to participate in voluntary studies often is rather low.

Transmission of the disease between farms, indicated by new positive herds, was detected in all the regions during the study period. Geographic clustering of BTM positive herds was seen in the southeast part of region Skåne (Figure 3). Skåne was the region where *M. bovis* was first diagnosed in Sweden in 2011, and there were several positive herds near that farm in southeast Skåne, suggesting transmission by local contacts. Unfortunately, we could not investigate this further due to lack of data on distances and status of neighboring farms.

In the present study, data from the DHI database were used to explore the associations between herd-level antibody status to *M. bovis* in BTM and PP cows and herd health and production variables. In this analysis, we believed it was better to use antibody status as positive or negative on herd level instead of antibody levels, because the outcome variables were retrieved at herd level. Introduction of cattle to the herd may be a risk factor for the health and production by introducing other infections and therefore we also included this variable in the analysis of health and production. We could not find any associations between herd-level antibody status and health and production in this study. This is in contrast to an earlier study where *M. bovis* antibodies in BTM was associated with higher late calf mortality, young stock mortality, and a tendency of reduced fertility as measured by proportion of cows with more than 120 d from calving to final insemination (Hurri et al., 2022). Other studies have also shown health effects associated with having *M. bovis* antibodies, such as a lower milk production (Uhaa et al., 1990a; Timonen et al., 2017) and reproduction disorders (Uhaa et al., 1990b). The reason that we could not see any health effects in *M. bovis*-positive herds in this study could be due to unspecific data for calves, we had information on mortality but not on treatments. This kind of study might need a larger dataset to see differences between herds. Production and reproduction parameters are partly depending on the farmer's strategy and decisions, for example on when to inseminate and when to cull, information that was not available in this study.

CONCLUSIONS

In this study we show that analyzing antibodies in milk from PP cows, in addition to BTM, is a useful strategy to find herds infected with *M. bovis*. Higher antibody levels in PP cows were associated with larger herd size and a higher number of introduced cattle. Sampling PP cows can facilitate finding the infection in an early stage, thus enabling prevention of transmission both within and between herds.

NOTES

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ish National Data Service (https://doi.org/10.5878/c45h-ke16). The animal study was reviewed and approved by the regional ethics committee in Uppsala, approval number 5.8.18-02650/2019. Written informed consent was obtained from the farmers for participation in this study. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: BTM = bulk tank milk; INHERDS = number of herds that the introduced cattle originated from; IQR = interquartile range; ÖG = Östergötland; PP = primiparous; SH = Swedish Holstein; SLU = Swedish University of Agricultural Sciences; SR = Swedish Red; VG = Västra Götaland.

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This thesis investigates *Mycoplasma bovis* infection in Swedish dairy herds, focusing on prevalence, epidemiology, and testing strategies. National screening showed regional prevalence (0-20%) and higher calf mortality in antibody-positive herds. Larger herd size and cattle introduction increased infection risk, and antibody-positive cows had lower milk production. Biosecurity practices, like affiliation to "Smittsäkrad besättning" program, were associated with a negative *Mycoplasma bovis* status. Adding individual cows to BTM testing improved herd detection. The findings suggest cost-effective control through biosecurity and monitoring.

Emma Hurri received her postgraduate education at the Department of Clinical Sciences, SLU, Uppsala. She obtained her veterinary degree from the Faculty of Veterinary Medicine, SLU, Uppsala.

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