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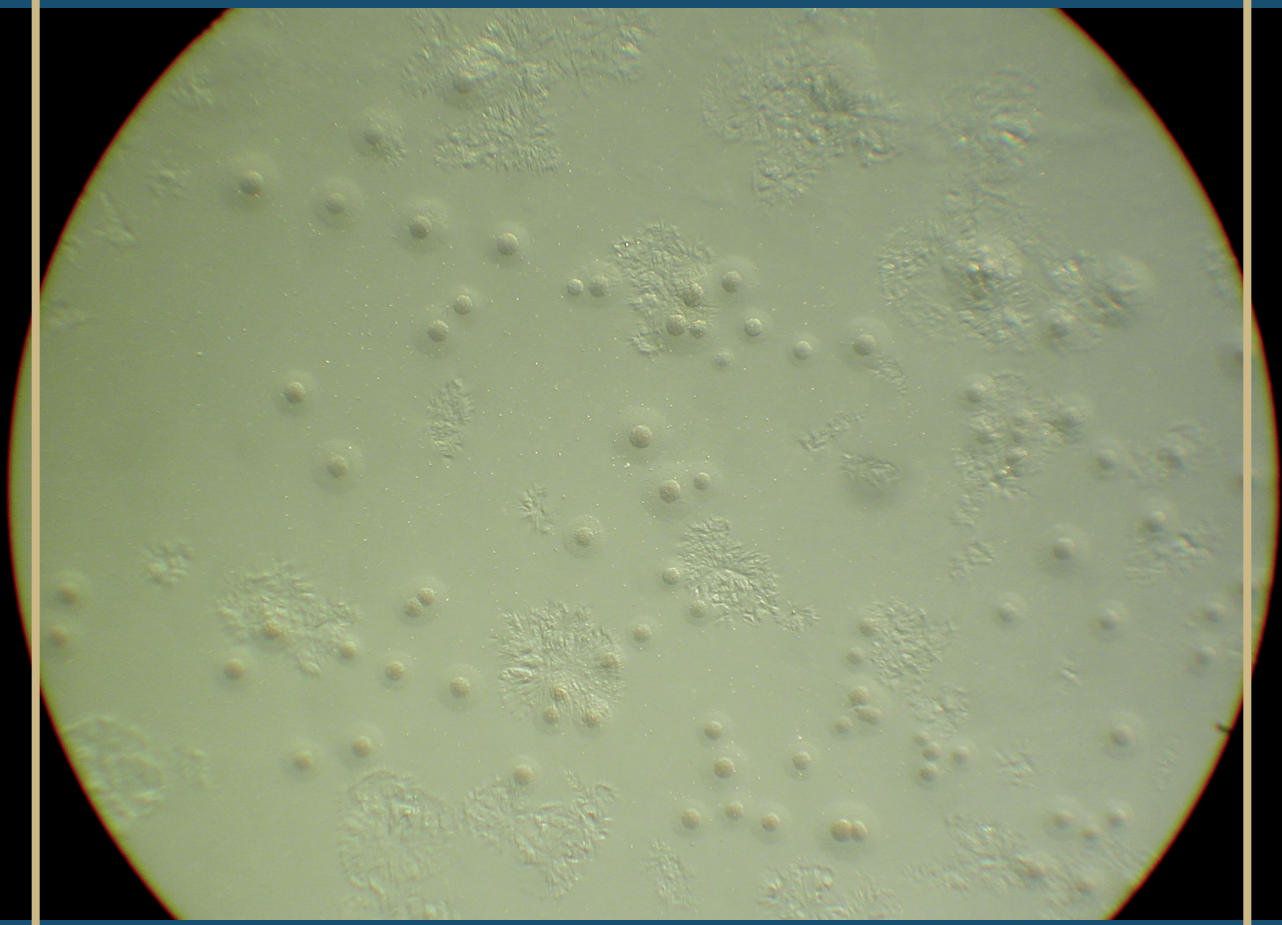
Tarja Pohjanvirta

Mycoplasma bovis in cattle: Studies on the transmission and control of infection



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MYCOPLASMA BOVIS IN CATTLE:

STUDIES ON THE TRANSMISSION AND CONTROL OF INFECTION

TARJA POHJANVIRTA

DOCTORAL DISSERTATION

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ABSTRACT

Mycoplasma bovis is an important pathogen of cattle. It causes a wide variety of clinical diseases, including mastitis, respiratory disease, arthritis, and otitis media. *M. bovis* has evolved several mechanisms to avoid the host's immune system, no effective commercial vaccines are available, and antibiotic resistance is increasingly seen in contemporary strains. The most common route of transmission between herds is the purchase of a latent carrier animal. *M. bovis* can spread silently within a herd, and once established in a dairy herd it is difficult to eradicate. Hence, it is of utmost importance to try to control and prevent the spread of *M. bovis*. Sensitive and cost-effective diagnostic methods to detect latent carrier animals, as well as methods that could be used in herd certification are needed. The possible transmission of *M. bovis* through assisted reproduction needs to be investigated more closely. This thesis examines the course of *M. bovis* infection in Finnish dairy farms, presents a new route of entry of *M. bovis* into naive dairy herds, and describes methods that could be utilized in the control of *M. bovis* infections.

In study I, the course of *M. bovis* infection was followed over two years on 19 recently infected dairy farms. The aim was to identify diagnostic methods that could be used to assess whether the herd had reached a low-risk infection status. In 17 herds, a few cases of clinical mastitis were detected, and these mostly occurred within two months after the index case. On two farms, *M. bovis* only caused respiratory disease in young stock, and no clinical mastitis was detected. The prevalence of *M. bovis* in nasal (NS) and deep nasopharyngeal (NP) swabs taken from young calves varied from zero to 75% per herd among the studied calves. An in-house MilA ELISA detected more positive serum samples than the commercial BioX ELISA. The proportion of MilA-positive young stock followed the patterns seen in NS and NP of calves, but no such association was seen in BioX ELISA results. In cows, despite the infection appearing to have resolved in some herds, the proportion of MilA antibody-positive cows remained high for at least one and half year after the detection of the index case. According to the results, *M. bovis* can be present in calves alone without causing mastitis in cows. Several methods need to be applied to verify the herd infection status. These include regular monitoring for *M. bovis* in clinical mastitis and calf pneumonia cases, combined with regular PCR testing of nasal swabs from calves and sera for the detection of antibodies against *M. bovis* using the MilA ELISA.

The transmission of *M. bovis* via semen has been speculated. When epidemiological data to assess the infection source were collected in study I, suspicion arose that contaminated commercial artificial insemination (AI) semen could have been the source in two closed dairy herds. National health care and

farm registers were used to collect farm data and farmers were interviewed. Whole genome sequencing was used to compare the genomes of isolated strains. Epidemiological analysis did not reveal any other source than contaminated semen from one bull used for artificial insemination in the cows that were the first ones to develop clinical mastitis in both herds. Core genome multilocus sequence type analysis supported this. The bull had secreted *M. bovis* intermittently and for only a short time into semen during an approximately seven-week period. On both farms, the incubation period between insemination and clinical mastitis was 32 days. To our knowledge, this is the first study to describe the transmission of *M. bovis* via AI. Even though this appears to be rare, commercial AI semen needs to be taken into account as a possible transmission route.

Following the detection of *M. bovis* in Finland, a voluntary control program was established. One part of the program is NS taken from young calves and tested for *M. bovis* to indicate the infection status of the herd. In study III, the suitability of this method was assessed and compared with NP sampling. Furthermore, NS and NP sampling of pneumonic calves to detect *M. bovis* was compared with bronchoalveolar lavage sampling. Altogether, 1037 NS were taken from calves in 30 recently infected herds, and NP samples were also taken from 284 calves. The overall prevalence in NS was 29.6% and the highest prevalence of 43% was seen in 31- to 60-day-old calves. Thereafter, the shedding rate decreased. At the calf level, NP sampling detected *M. bovis* in 47% and NS in 33% of studied calves. At the herd level, NS sampling was more sensitive, as it classified 51 out 54 herd visits with a positive infection status as infected, whereas using NP sampling, the respective figure was 43 out of 54 visits ($p = 0.061$). The reason for the difference seen at the calf and herd levels is the sampling protocol. We took only five NP samples, but number of NS swabs varied from six to 28, depending on the herd size. We conclude that NS swabs taken from calves under six months of age and analyzed with real-time PCR represent a cost-efficient method to be used in a control program. If calves suffering from acute respiratory disease need to be examined, NP samples are a practical and sensitive method to detect *M. bovis*.

The effect of two concentrations of a gentamycin-tylosin-linco/spectinomycin (GTLS) antibiotic combination and a fluoroquinole antibiotic, ofloxacin, on the viability of *M. bovis* in commercial-scale AI semen production using modern semen extender with plant-derived protein was investigated. A reference strain and a wild-type strains isolated from semen in study II were used in spiking. Three different protocols to extract *M. bovis* DNA from semen were also compared. At a high spiking concentration of 10^6 CFU/mL, none of the studied antibiotics had a bactericidal effect. At a low spiking concentration of 10^3 CFU/mL, the growth of the wild-type strain was inhibited by all other antibiotic protocols except for the low GTLS concentration, which is stated in EU regulation and the OIE Terrestrial Code. Instead, the high GTLS protocol was the only one that inhibited the growth of the reference strain. At a low *M. bovis* contamination level, GTLS used at a high

concentration, according to the Certified Semen Services protocol, is more efficient than GTLS used according to the OIE Terrestrial Code. The Instagene™ matrix was the most efficient method to extract *M. bovis* DNA from semen.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals. In addition, some unpublished results are presented.

I Characterisation of the course of *Mycoplasma bovis* infection in naturally infected dairy herds. Vähänikkilä N, Pohjanvirta T, Haapala V, Simojoki H, Soveri T, Browning GF, Pelkonen S, Wawegama NK, Autio T. Vet Microbiol. 2019 Apr; 231:107–115. doi: 10.1016/j.vetmic.2019.03.007

II Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. Haapala V, Pohjanvirta T, Vähänikkilä N, Halkilahti J, Simonen H, Pelkonen S, Soveri T, Simojoki H, Autio T. Vet Microbiol. 2018 Mar; 216:60–66. doi: 10.1016/j.vetmic.2018.02.005

III Suitability of Nasal and Deep Nasopharyngeal Swab Sampling of Calves in the *Mycoplasma bovis* Control Program. Pohjanvirta T, Vähänikkilä N, Talvitie V, Pelkonen S, Autio T. Front Vet Sci. 2021 Sep 10;8:689212. doi: 10.3389/fvets.2021.689212

IV Efficacy of Two Antibiotic-Extender Combinations on *Mycoplasma bovis* in Bovine Semen Production. Pohjanvirta T, Vähänikkilä N, Simonen H, Pelkonen S, Autio T. Pathogens. 2020 Sep 30;9(10):808. doi: 10.3390/pathogens9100808

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ABBREVIATIONS

adh-1	Alcohol dehydrogenase -1 gene
AI	Artificial insemination
BAL	Broncho-alveolar lavage
BRD	Bovine respiratory disease
BTM	Bulk tank milk
cgMLST	Core genome multilocus sequence typing
CSS	Certified Semen Services, a subsidiary of the National Association of Animal Breeders, USA
ELISA	Enzyme-linked immunosorbent assay
GTLS	Gentamycin-tylosin-lincomycin-spectinomycin
IFN-γ	Interferon gamma
IL	Interleukin
LRT	Lower respiratory tract
MIB-MIP	Mycoplasma immunoglobulin binding–mycoplasma immunoglobulin protease
MilA	Mycoplasma immunogenic lipase A
NET	Neutrophil extracellular trap
NGS	Next-generation sequencing
NP	Deep nasopharyngeal swab
NS	Nasal swab
ODC	Optical density coefficient
OIE	World Animal Health Organisation
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PDL-1	Programmed cell death ligand 1
PG45	*M. bovis* international reference strain Donetta PG45, ATCC 25523
QMS	Quarter milk sample
rtPCR	Real-time polymerase chain reaction
Se	Test sensitivity
Sp	Test specificity
Th1/2	T helper cell type 1/type 2
URT	Upper respiratory tract
wgMLST	Whole genome multilocus sequence typing
WGS	Whole genome sequencing
wgSNV	Whole genome single nucleotide variant
Vsp	Variable surface protein

1 BACKGROUND

Mycoplasma bovis (*M. bovis*) was first isolated in the USA in 1961 from a mastitis outbreak [1]. During the following decades, it spread to other continents and was regarded as endemic in Europe by the end of the century, except in Norway and Finland [2,3]. *M. bovis* causes a plethora of diseases in cattle, including pneumonia, mastitis, arthritis, otitis media, and genital disorders [4]. *M. bovis* infections tend to be chronic and reduce animal welfare, they respond poorly to antibiotic treatment, and no effective commercial vaccine exists [4,5]. Thus, prevention and control of the spread of infection is of utmost importance.

In Finland, two studies applying mycoplasma culture to BAL samples from non-medicated calves suffering from acute respiratory disease were conducted in 1998–1999 and 2002–2004 [6,7]. No *M. bovis* was found. *M. bovis* usually enters a naive herd through a latent carrier animal bought into the herd. The number of cattle annually imported to Finland is small and breeding animals for suckler cow herds are mainly imported [8]. The association Animal Health ETT has instructed importers to test animals considered for import for *M. bovis* antibodies if *M. bovis* is known to be endemic in the country of origin. Passive surveillance for *M. bovis* has been conducted in Finland since 2004. All bovine post-mortem samples in which pneumonia has been suspected, as well as bovine fetuses sent for post-mortem examination, and NP samples taken from calves with acute BRD have been cultured for *M. bovis* in the laboratories of Finnish Food Authority with negative results.

Laboratory analysis of QMS to detect mastitis pathogens using rtPCR is widely applied in Finland. In spring 2012, mastitis laboratories started to use a commercial rtPCR kit including primers and a probe specific for *M. bovis* (Pathoproof Mastitis Complete 16, Thermo Fisher Scientific, Vantaa, Finland). A calf-rearing farm with approximately 500 calves had, according to the calf caretakers, experienced respiratory disease responding poorly to antibiotic treatment since spring 2012, and some calves had droopy ears and head tilt. It was not until the end of October that they took NP samples for laboratory diagnosis, and we found the bacterium that for 10 years we had been looking for and hoping not to find. Soon after, on a small farm raising bull calves for meat production, a devasting outbreak of respiratory disease caused by *M. bovis* together with *Histophilus somni* occurred. Later, in December, a herd with 20 milking cows experienced a mastitis outbreak that led to the culling of four cows. Using rtPCR and culture, *M. bovis* was detected as the sole pathogen causing mastitis in three cows and together with *Staphylococcus aureus* in one cow.

This sudden appearance of *M. bovis* in Finland led to the rapid establishment of antibody ELISA testing, as well as in-house rtPCR to detect *M. bovis* in our laboratory. Hundreds of serum samples from the infected farms and their contact farms, as well as sera from all imported animals from 2010 to 2012 and suckling beef cattle slaughtered in early 2012 (assumed to be a totally negative population), were tested for anti-*M. bovis* antibodies using the only commercial ELISA tests available in Europe at that time. This soon revealed that the results were contradictory to the clinical picture seen in tested animals. On several farms, one or two positive test results without any clinical signs in the herd were detected. Help was sought from the Mycoplasma Laboratory of the Animal and Plant Health Agency (APHA) in the UK. In APHA, antibody ELISA whole cell antigen is used instead of unknown recombinant protein expressed in *E. coli* used in the commercial ELISA. Again, quite contradictory results were obtained from the same serum samples. Animal Health ETT and the cattle industry wanted to prevent the uncontrollable spread of *M. bovis*. A national voluntary control program was established during 2013 (described in chapter 3.1.5). As the antibody testing results were contradictory and we suspected that the commercial ELISA had low sensitivity, rtPCR examination of NS samples from 1-week-old to 6-month-old calves was instead included as one part of the control program based on the findings reported by Bennett and Jasper [9].

A project aiming to gain knowledge of the epidemiology and nature of infection on dairy farms, to improve sampling and diagnostic measures, and to examine measures aiming at the eradication of *M. bovis* from the infected herds was started at the beginning of 2014. During this project, a suspicion arose that commercial semen had been the source of infection in two closed dairy herds. Because there was a lack of research regarding *M. bovis* survival in modern commercial semen and embryo production, another project was started. In this project, we analyzed *M. bovis* survival in commercial-scale semen production using different antibiotic combinations and compared different DNA extraction methods to detect *M. bovis* DNA in semen. This thesis describes the course of infection on Finnish dairy farms, a new route of entry of *M. bovis* into naive herds, and methods that can be utilized in the control of *M. bovis* infections.

2 REVIEW OF THE LITERATURE

2.1 An overview of *M. bovis*, *M. bovis*-associated diseases, and immune responses to *M. bovis* in cattle

2.1.1 Characterization of *M. bovis*

Mycoplasmas belong to the bacterial class *Mollicutes*. The literal meaning of this Latin word is soft skin, which describes the lack of a cell wall in mycoplasmas. They evolved from an ancestor of Gram-positive bacteria by genome reduction [10] and are considered to be one of the fastest evolving bacteria [11]. Due to their small genome size of approximately 0.6 to 1 kbp, mycoplasmas have limited metabolic pathways and rely on their host for the acquisition of many essential nutrients. The genus *Mycoplasma* includes over 100 species, out of which 13 are known to infect cattle, although with varying degrees of clinical importance [12]. *Mycoplasma mycoides* subsp. *mycoides*, the causative agent of contagious bovine pleuropneumonia, is the only bovine mycoplasma included in the OIE list of animal diseases [13]. Contagious bovine pleuropneumonia has been eradicated from most continents, but still exists in several African countries [14]. *M. bovis* is globally widespread and is economically the most important mycoplasma species infecting cattle [4]. Other *Mycoplasma* species of interest in cattle include *M. alkalescens*, *M. arginini, M. bovigenitalium*, *M. bovirhinis*, *M. bovoculi, M. californicum, M. canadense, M. dispar, M. canis, M. leachii*, and *M. wenyonii* [12] .

When *M. bovis* was first isolated from a mastitis outbreak in the USA, it was tentatively given the name *M. agalactiae* var. *bovis* [1] due its close resemblance to *M. agalactiae* in small ruminants. However, in 1976, Askaa and Ernø reported a low DNA–DNA hybridization value of 38% between *M. agalactiae* and PG45-type strains [15]. This warranted a species status, and *M. bovis* has since been the species name. The genome size of *M. bovis* varies from approximately 0.948 to 1.003 kbp and the guanine plus cytosine content is on average 29.3%.

The pathogenesis of *M. bovis* is not completely understood and a number of factors appear to play a role in its virulence [16,17]. Because of the paucity of metabolic capacity in *M. bovis*, adhesion to host cells plays an important role in virulence. Several possible adhesins binding to host extracellular matrix components, such as fibronectin, plasminogen, and heparin, have been described in *M. bovis* [18–27], and some of them appear to be multitasking proteins [18,23,28]. Some nucleases have been shown to associate with escape from NETs or cytotoxicity [29-31]. *M. bovis* produces H_2O_2, which in turn causes toxic damage to host cells [32,33]. However, there appears to be large variation in H_2O_2 production between strains [32], and the importance of H_2O_2 production in pathogenesis still

needs clarification. Secondary metabolites, namely reactive oxygen and nitrogen species released by phagocytes attracted to the infection site, also appear to be important in *M. bovis* pathogenesis [33,34]. Intracellular invasion of *M. bovis* into several cell types has been described [35-41], and this phenomenon can protect bacteria from elimination by secreted antibodies and therapeutic antibiotics.

2.1.2 *M. bovis*-associated diseases

M. bovis is one of the infectious agents in the BRD complex and a causative agent of bovine contagious mastitis. Arthritis can be seen in conjunction with mastitis and pneumonia, and in calves, respiratory infection is often accompanied by otitis media [4]. Various reproductive disorders connected to *M. bovis* have been described, such as abortion and seminal vesiculitis ([42-44]. Other less frequent clinical conditions caused by *M. bovis* have been reported, such as endocarditis [45], post-surgical seromas [46], keratoconjunctivitis [47,48], brain tissue infection [49,50], meningitis [51], and decubital abscesses [52].

2.1.2.1 Mastitis

Mastitis caused by *M. bovis* is portrayed as being highly contagious, usually affecting more than one quarter, causing a significant decrease in milk production, and not responding to antibiotic therapy. Affected cows are usually non-febrile and alert. Milk consistency can be watery, with fine particles that form sediment [53,54]. *M. bovis* mastitis has also been described in dry cows [55,56] and heifers [57]. Experimental studies have demonstrated that intramammary *M. bovis* infection causes a similar rise in the milk somatic cell count as seen in mastitis caused by *E. coli* or *S. aureus*, but in contrast, the bacterial counts in milk are extremely high, from 10^8 to 10^{10} CFU/mL [58-60]. Based on the field experience of many investigators, clinical *M. bovis* mastitis is considered to be untreatable and culling is recommended [54,61].

2.1.2.2 Bovine respiratory disease (BRD)

M. bovis has emerged as an important infectious agent in BRD [50,62]. The development of BRD is a complex network in which host factors, such as age, the maturity of the immune system, and the composition of the URT microbiome, and environmental factors, such as temperature, stocking density, transportation and commingling, and different combinations of bacteria and viruses, all affect the outcome [63,64]. *M. bovis* can be a frequent colonizer of the URT in young calves in endemic areas [65], and it has been found in LRT samples from healthy calves [66]. In pneumonia, *M. bovis* is nearly always detected with other pathogens [67] and can have a synergistic role, exacerbating the disease [68]. In studies on the bovine respiratory tract microbiota of feedlot calves, *M. bovis* was significantly more often detected in NP samples from BRD calves (44%) compared to healthy

controls (18%), but the relative abundance of bacteria did not differ between groups [69]. One form of pneumonia, namely caseonecrotic pneumonia with or without arthritis, is known to be specifically caused by *M. bovis* [63,70,71]. Pneumonia in which *M. bovis* is involved can occur in cattle of almost any age and in all cattle sectors but is most common in intensively managed beef and veal calf feedlots in calves younger than six months old [63,67,72-78]. In dairy calves, *M. bovis* was shown to be associated with clinical BRD signs, lung consolidation, and low daily weight gain [79]. Less is known about the importance of *M. bovis* in pneumonia in cows. In Brazil, *M. bovis* was frequently detected in tissues from cows with fatal pneumonia [80].

2.1.2.3 Arthritis

M. bovis-associated arthritis is thought to occur after hematogenous spread from the lungs or udder to joints and is seen in both calves and cows. Animals with *M. bovis* arthritis become acutely lame, displaying swollen joints and tendon sheaths [51,70,71,81-85]. *M. bovis* arthritis is painful and responds poorly to antibiotic therapy, often leading to the culling of infected animals [63,82,83]. According to Jensen [86], arthritis was the most common form of *M. bovis*-associated disease in Danish dairy farms in a recent outbreak. It was also noticed that recovery was possible under field conditions.

2.1.2.4 Otitis media

Otitis media is frequently observed in young calves with *M. bovis* respiratory disease. Clinical signs include head shaking, head tilt, and uni/bilateral ear droop. The middle ear is filled with fibrinosuppurative to caseous exudate. Otitis media can progress to otitis interna and meningitis [37,87-91]. Infection arises from the nasopharynx via the Eustachian tube to the middle ear and feeding with contaminated milk has especially been shown to promote the colonization of the tonsils and Eustachian tube [92,93].

2.1.2.5 Reproductive disorders

Less is known about the role of *M. bovis* in bovine reproductive disorders. Hartman [94] inoculated a culture of "Connecticut mammary pathogen" [1], that is, *M. bovis*, into the uterus of eight heifers. Neutrophilia and mucopurulent vaginal discharge were observed in some animals, but no clinical signs were otherwise noted. Endometritis, salpingitis, and salpingoperitonitis of varying degrees were seen at necropsy. One animal that was followed for 100 days secreted *M. bovis* in vaginal mucus 64 days after inoculation. Hirth et al. [95] inseminated heifers with semen contaminated with *M. bovis*. Almost all the heifers became repeat breeders and post-mortem suppurative salpingitis, chronic endometritis, and ovarian adhesions were seen in some animals. In the contaminated semen, *M. bovis* was shown to be viable after 18 months of storage in liquid nitrogen. Pfüzner and Schimmel

[96] cultured various organs of cows with *M. bovis* mastitis after slaughter and isolated *M. bovis* in 10% of uterus samples. They also observed that *M. bovis* was occasionally transmitted *in utero* to the fetus and newborn calf. However, apart from this report, isolations from aborted fetuses have infrequently been made [44,97]. Sometimes, during an acute *M. bovis* mastitis outbreak, farmers have reported an increased incidence of abortions, but no microbiological investigations have been conducted to clarify the role of *M. bovis* in these abortions [56]. In bulls, mycoplasmas appear to reside in the preputium and distal urethra and may contaminate semen [98,99]. Experimental inoculation of *M. bovis* into the prepuce or urethra was shown to cause seminal vesiculitis and orchitis and low semen quality, and shedding of *M. bovis* in semen was detected [100].

2.1.2.6 Prevalence and economic impact

Very diverse prevalences of *M. bovis*-associated diseases have been reported, because different diagnostic methods and samples have been used in prevalence estimations. In Europe, when calves arrive at feedlots, the prevalence of *M. bovis* is generally low (0% to 7%), but within one month it is usually from 40% to 100% [77,101]. Prevalence studies in dairy calves are scarce. Fanelli et al. [102] detected *M. bovis* in fatal dairy calf pneumonia in 26.6% of studied herds with an animal prevalence of 16% and as the only pathogen in more than half of the cases. Herd prevalences of *M. bovis* investigated with different methods in European countries are presented in Table 1.

Table 1. Herd prevalence of *M. bovis* in different European countries.

Country	Prevalence %	Sample/method	Herd type	Reference
Belgium	1.5	BTM PCR	Dairy	[103]
Belgium	11	Serum ELISA	Veal	[104]
Belgium	7/17	BTM PCR/ELISA	Dairy	[105]
Denmark	1.6/7.2	BTM PCR/ELISA	Dairy	[106]
Denmark	2.9	BTM ELISA	Dairy	[107]
Denmark	9.6	NS PCR	Dairy	[108]
Estonia	48.3	Serum ELISA	Dairy	[109]
France	60	Serum ELISA	Veal	[72]
France	0	BTM PCR	Dairy	[110]
Greece	5.4	QMS culture	Dairy	[111]
Hungary	100	Serum ELISA	Fattening	[112]
Ireland	0.42/30*	BTM ELISA	Dairy	[113]
Norway	0	Serum ELISA	Dairy	[3]
Poland	65.5	Serum ELISA	Fattening	[114]
Portugal	2.4	BTM PCR	Dairy	[115]
Sweden	0–0.3	BTM PCR	Dairy	[116]

*Year 2019/2020, the same ELISA test

In Finland, during the last ten years, *M. bovis* has been detected on 102 dairy farms and 231 fattening cattle farms (Fig. 1). These numbers are based on the detection of *M. bovis* in samples sent to the Finnish Food Authority as diagnostic samples or samples sent from other laboratories for confirmation.

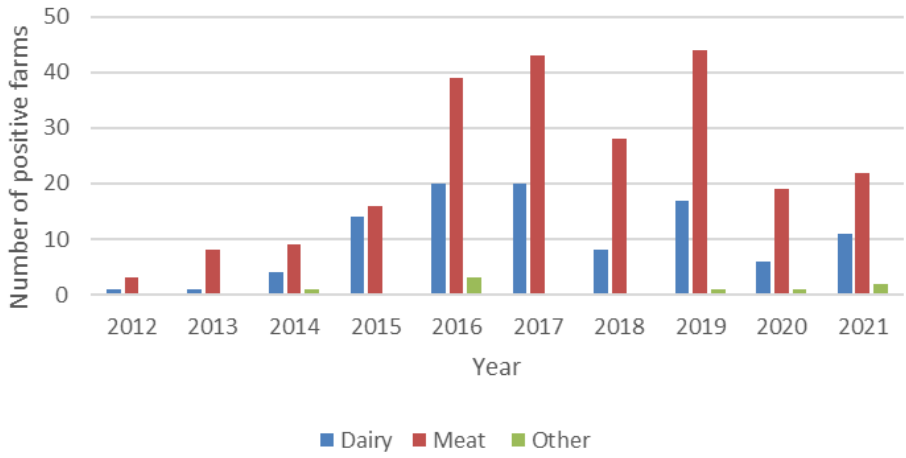

Figure 1. The number of farms on which *M. bovis* has been detected in clinical samples during 2012–2021 in Finland. Each farm has been counted only once.

Studies assessing the economic impact of *M. bovis* infections have only been published before the 21st century. Production losses for American dairy industry were then estimated to be 108 million dollars per year[117]. Financial losses due to antimicrobial therapy, extra labor, mortality, culling, and diagnostic tests were not included in this study. In Europe, 576 million euros per year were reported to be lost due to BRD, of which 25% was attributable to *M. bovis* [2]. Production animal disease experts around the world ranked *M. bovis* infections in cattle among the most important enzootic diseases when the EU database DISCONTOOLS was founded, highlighting its importance to the economy and cattle welfare [118]. *M. bovis* also causes subclinical costs in production, but these have not properly been analyzed. Petersen et al. [119] demonstrated that heifers originating from herds with a high seroprevalence against *M. bovis* had an increased risk of early unwanted departure from the dairy herds.

2.1.3 Host immune responses to *Mycoplasma bovis* infection

The bovine innate immune system reacts strongly to *M. bovis* infection, and both the T cell-mediated cellular and B cell-mediated humoral response of the adaptive immune system are activated. *M. bovis* has in its arsenal several mechanisms to dampen the immune response, which often leads to chronic infections and makes *M. bovis* a successful pathogen. The immune response to *M. bovis* infection in

cattle is complex and currently under extensive investigation. It is beyond the scope of this thesis to describe immune responses in detail, but some of the main mechanisms are portrayed here. In a broad sense, *M. bovis* employs four different types of mechanisms to overcome the bovine immune system: antigenic variation, location intracellularly or in a biofilm, mechanisms to circumvent the effector molecules of the immune system, and modulation of the immune system to downregulate it.

M. bovis possesses a large repertoire of highly immunogenic variable surface lipoproteins, or Vsps. Some of these are important in host cell adhesion [19,120]. Vsps have been best studied in the PG45 strain, which has an operon capable of encoding 13 different Vsps [121-124]. The expression of Vsps undergoes high-frequency phase variation (ON/OFF), and the size of the expressed Vsp can vary [122,125-129]. When the host starts to produce antibodies against a Vsp, *M. bovis* switches expression to another type and thus avoids opsonization [130]. This reduces mycoplasma killing by complement activation and phagocytosis by neutrophils and macrophages. Interestingly, no *vsp* genes have been detected in two Chinese strains, Hubei-1 [131] and CQ-W70 [132], but Hubei-1 is known to express another type of surface lipoprotein called VpmaX [22].

Intracellular residence, which protects *M. bovis* from attack by the host immune system, has been demonstrated in many cell types of bovine PBMCs, and to a lesser extent in erythrocytes [38] and in some bovine epithelial cell lines [133]. *M. bovis* can form biofilm *in vitro*, although some strain variation occurs [134]. Biofilms protect bacteria from stressful conditions such as heat, desiccation, and even antibiotics. However, in the study by McAuliffe et al. [134], no significant variation in MICs compared to planktonic cells was seen. It is not known whether *M. bovis* forms biofilm during natural infection in the host.

Neutrophils are important cells of the innate immune system and are the most abundant immune cell type recruited to the lungs, middle ear, joints, and mammary gland during *M. bovis* infection [35,37,59,71,135]. Neutrophil extracellular traps (NETs) are extracellular fibers mainly composed of neutrophil DNA and some granules, and they allow neutrophils to kill extracellular pathogens [136]. *M. bovis* induces the formation of NETs, but these are rapidly degraded by an extracellular nuclease [31]. *Mannheimia haemolytica* and *Histophilus somni*, which are common pathogens in the BRD complex together with *M. bovis*, also induce NET formation [137,138]. It has been speculated that the production of nuclease degrading NETs is a way for *M. bovis* to increase its own survival in inflamed lung tissue when other bacterial pathogens are involved. Many mycoplasmas have been demonstrated to have an Ig-binding and Ig-cleaving system, MIB–MIP [139,140]. This system promotes the dissociation of the antigen–antibody complex because Ig protease cleaves the Ig heavy chain [141]. The MIB–MIP system has also been found in *M. bovis* PG45 [142]. Further studies are still needed to resolve the exact mechanism of this system in natural infections. By degrading immunoglobulins that attach to

their surface, mycoplasmas can inhibit complement activation and phagocytosis, as well as some functions of the adaptive immune system [141].

Other mechanism by which *M. bovis* downregulates host immune mechanisms include delaying apoptosis in monocytes [143] and macrophages [144,145]. This is expected to reduce the effector functions of these cells and alter the immune response [146]. A substantial body of evidence has been gathered to show that *M. bovis* causes a phenomenon called T cell exhaustion [147-149]. T cell exhaustion is characterized by the surface expression of immunoinhibitory receptor PD-1, and its ligand, PD-L1, is widely expressed on lymphocytes and phagocytes. This co-expression inhibits T cell replication and normal effector functions, including memory-T cell responses [146]. Prostaglandin E2 has been demonstrated to induce PD-L1 expression in bovine PBMCs. T cell exhaustion is reversible and can be ameliorated with anti-PD-L1 antibody. Meloxicam, one of the most common non-steroid anti-inflammatory drugs used in cattle, inhibits prostaglandin synthesis. Together with PD-L1 antibody, meloxicam has been observed to further enhance the restoration of lymphocyte effector functions [149]. Biopharmaceuticals that block the PD-1/PD-L1 pathway are already used in human medicine, and such compounds, together with prostaglandin inhibitor, might have potential for controlling *M. bovis* infections.

M. bovis respiratory infection and mastitis usually elicit a strong humoral immune response. Experimental intratracheal infection of gnotobiotic calves led to an IgM antibody response after one week and an IgG response within two weeks in serum, and to an IgA response in tracheal washings. IgG1 was the predominant isotype, but some IgG2 was also detected [150]. Van den Bush et al. [151] challenged 12-week-old calves intratracheally. An IgG response was clearly seen 28 days post-infection. They also analyzed the cellular response in PBMCs and found that the percentage of cells producing IFN-γ and IL-4 was equal. However, IgG1 was the dominant isotype and very little IgG2 production was detected during a 9-week post-challenge follow-up. IFN-γ is secreted by Th1 cells, whereas IL-4 is the hallmark cytokine for Th2 activation. IL-4-secreting Th2 cells are efficient B cell helpers and IL-4 drives B cells to secrete IgG1 [152]. Thus, based on their study, Vanden Bush et al. [151] suggested that the immune response towards *M. bovis* has a Th2 bias. Similarly, Hermeyer et al. [43] found that IgG1-producing plasma cells were predominant in the lungs of calves with pneumonia. Howard [153] demonstrated that in cattle, IgG2 is a superior opsonin compared to IgG1, and a low IgG2 response is therefore probably one factor that contributes to the chronicity of *M. bovis* infections. In an experimental mastitis vaccination study, adult cows produced both systemic and milk IgG1 and IgG2 responses, suggesting that in mature animals, the immune response is less Th2 skewed [154].

There is very little information in the literature about maternal antibodies to *M. bovis*, even though these can affect the serological testing of young calves

and vaccination success. Tschopp et al. [76] stated that 39% of calves born to seronegative cows had maternal *M. bovis* antibodies and that the half-life of the maternal antibodies was 20 days. However, this study was based on a relatively small number of 20 cows and their offspring. Arcangioli et al. [73] reported that 2.2% of veal calves in France had maternal antibodies at the time of assembly. These antibodies appear to have little protective effect, as in 12- to 41-day-old veal calves, seropositivity against *M. bovis* had no protective effect against BRD [155].

Recent studies on the prevalence and dynamics of *M. bovis* antibodies in the serum and milk of cattle are reviewed in chapter 2.3.2.3.

2.2 Transmission

2.2.1 Sources and uptake of *M. bovis*

Known sources for *M. bovis* are diseased or latent carrier animals, milk, and the milking environment [4,156]. The role of colostrum, a contaminated farm environment, fomites, and assisted reproduction in *M. bovis* transmission is less clear.

Under field conditions, animals become colonized/infected via inhalation, ingestion, or invasion through the teat canal. The risk of *M. bovis* infecting an animal through the genitourinary mucosa under field conditions is not known. Aerosols and nose-to-nose contact with an infected animal are considered to be important sources of infection [4]. Calves can be infected through aerosolized *M. bovis* culture [68,157,158]. *M. bovis* has been cultured from air in a barn holding sick animals [159], but in later research, Soehnlen et al. [160] could not detect *M. bovis* in the air of a veal calf barn in which most of the calves were infected. The importance of airborne between-herd transmission is well recognized for *M. hyopneumoniae* on swine farms. This mycoplasma has been shown to infect farms over a distance of 9 km [161,162], but to author's knowledge, no reports of the airborne transmission of *M. bovis* between herds have been published.

In cows, udder-to-udder transmission during milking via a milking machine, udder wash cloths, or the milker's hands is one common transmission route [53,54]. In calves, feeding with contaminated milk is one of the main infection sources [92]. Colostrum has been suggested as one source of *M. bovis* for calves [87,90], but no research to prove this has been published. Using rtPCR, Gille et al. [163] detected *M. bovis* in colostrum samples in four out of 17 studied herds, but the C_t values indicated that the number of bacteria was low in the samples. Timonen et al. [164] estimated the prevalence of *M. bovis* in colostrum in four Estonian herds to vary from 1.7% to 4.7%, but they did not study the infectivity of colostrum. Nevertheless, pasteurization of colostrum in infected dairy herds to destroy mycoplasmas has been recommended [165].

Despite its simple structure and need to gain nutrients from the host, *M. bovis* has been shown to survive in the environment, especially at a low temperature under humid conditions and when protected from light. Pfüzner [166] reported the survival of *M. bovis* at 4 °C to be almost two months in milk and sponges and two weeks in water. Schibrowski et al. [167] demonstrated that a shared water bucket was a highly significant risk factor for *M. bovis* infection in Australian feedlot pens. Justice-Allen [168] reported survival in recycled sand bedding to be several months, although Wilson et al. [169] could not show any infectivity via contaminated sand in naïve calves kept on sand bedding. Piccinini et al. [170] found that *M. bovis* survived on the surfaces of cages and mangers, and the environment could have been the source of infection for veal calves. In New Zealand, it has been noticed that live animals and contaminated milk form a high-risk source for *M. bovis* transmission. Milking equipment and other materials from the milk handling environment are considered as medium risk, whereas lanes and water troughs outside, for example, are low-risk sources [171]. Whether disinfection of the barn is an important protective factor against *M. bovis* reinfection is not clear, but it is mandatory in New Zealand in infected premises after depopulation and cleaning.

The role of assisted reproduction as a source of *M. bovis* is unclear. The possible risks of transmitting *M. bovis* via semen or embryo transfer are discussed in depth in the review of Wrathall et al. [172], who concluded that some risk exists. Few studies have demonstrated the presence of *M. bovis* in the semen or preputium of bulls. Amram et al. [173] cultured *M. bovis* from commercial AI semen in Israel. Jain et al. [174] examined 12 bull semen samples in India using rtPCR and detected *M. bovis* in 27% of the samples. Kirchhoff and Binder [175] collected 182 semen samples and 210 preputial wash samples from normal bulls in Germany. *M. bovis* was identified in one of the preputial samples but not in semen. In this study, two bulls displayed clinical signs of epididymitis, and *M. bovis* was isolated from semen samples and one preputial wash. Trichard and Jacobsz [176] collected both preputial and semen samples from AI centers and privately owned bulls in South Africa. *M. bovis* was detected in 0.5% of both sample types. However, apart from the study of Amram et al. (2013), the specificity of methods used in other studies has not been described. Several other studies have investigated raw and processed semen, as well as preputial samples, for the presence of *M. bovis* without detecting it [99,177-180]. Bielanski et al. exposed *in vitro*-produced embryos during culture to *M. bovis* [181] or used contaminated semen in fertilization to produce *in vitro* embryos [182]. They concluded that *M. bovis* adheres tightly to the *zona pellucida* but does not affect embryo development. *M. bovis* present in semen can be transmitted through *in vitro* embryo production, because even washes carried out according to the instructions of the International Embryo Transfer Society do not render embryos free of *M. bovis* [182]. Thus, embryos could be a source of *M. bovis* infection, but to the author's knowledge, no studies have been published in which *M. bovis*-contaminated embryos have been transferred to recipient cattle.

Mycoplasmas are usually very host specific, and the risk of other animals transmitting *M. bovis* to cattle is likely to be low [183]. *M. bovis* has occasionally been detected in other animal species, like in small ruminants in the UK [50], in pigs grazing on Alpine pastures together with cattle [184], in broiler chickens on a farm also holding cattle [185], and in farmed white-tailed deer (*Odocoileus virginianus*) suffering from pneumonia [186].

2.2.2 Excretion of *M. bovis*

The respiratory tract and udder are the most important sites for the colonization and shedding of *M. bovis* [4,187], but the colonization of other body sites, such as the conjunctiva and vagina, has also been described ([188-192]. The risk of shedding from animals only displaying arthritis has not been investigated. Stressful events such as transportation, overcrowding, commingling, parturition, and unfavorable weather conditions have been associated with increased shedding and outbreaks of *M. bovis*-associated diseases [82,193,194]. Alabdullah [195] mimicked the effect of stress by administering dexamethasone, a synthetic glucocorticoid, to calves and examining its effect on *M. bovis* shedding. His results confirmed that stress is likely to influence *M. bovis* shedding, as calves treated with dexamethasone were found to shed *M. bovis* significantly more often compared to controls. Furthermore, the duration of shedding was longer in dexamethasone-treated calves than controls.

Both diseased and healthy carriers can shed bacteria into nasal secretions. Bennett and Jasper [9] demonstrated that in calves younger than 9 months old, the within-herd prevalence of nasal shedding was higher in dairy herds with *M. bovis*-associated disease (34%) compared to herds with no such history (6%). The duration of shedding after clinical pneumonia has not been elucidated. Cows with mycoplasma mastitis tend to have an inconsistent daily shedding pattern [187,196]. According to Punyapornwithaya et al. [188], an *M. bovis* strain that had caused clinical mastitis in a dairy herd colonized multiple body sites of cattle, including the nose, conjunctiva, and vulvovaginal tract, over one year after the last mastitis case. However, the prevalence of colonization decreased markedly over time. Hazelton et al. [190] found a low percentage of vaginal colonization and no nasal colonization in cows recently diagnosed with *M. bovis* mastitis. In a 2-year longitudinal study starting with 450 heifer calves, Hazelton et al. [191] found that at weaning, 3.6% of the calves were colonized with *M. bovis,* mainly shedding it into nasal secretions. However, only one out of 356 animals that calved was found to be shedding *M. bovis* into nasal secretions post-calving. After an *M. bovis* mastitis outbreak, *M. bovis* can persist in the herd as subclinical intramammary infections. However, Hazelton et al. [197] studied four large Australian dairy herds in which all cows with clinical *M. bovis* mastitis had been culled and only found

subclinical IMI in two out of the four herds. The prevalence of subclinical IMI was very low in these two herds, namely 0.1% and 0.2%.

These studies have demonstrated that shedding of *M. bovis* can be intermittent, very few animals in the herd might be latent carriers, and no consistently infected and easily accessible anatomical site for sampling has been found. These facts pose serious problems in reliably detecting carrier animals.

2.3 Detection and molecular epidemiology

2.3.1 Specimen selection for *M. bovis* detection

Among the essential steps for successful disease control are evaluation of the sampling methods and diagnostic tests, as well as how to use these in prevalence studies. Specimen selection for *M. bovis* detection depends on the purpose of sampling, i.e., identifying the causative agents in an acute disease outbreak and possibly needing an antibiotic sensitivity test, trying to detect carrier animals, or disease surveillance. Different sample types and their potential use in the above-mentioned situations are listed in Table 2.

Table 2. Description of different sample types and detection methods that can be used in *M. bovis* detection.

Sample type	Applied test	Age group	Application
Nasal swab	PCR	Calves	Detecting carriers
Deep nasopharyngeal swab	Culture, PCR	Calves	Respiratory disease diagnostics, detecting carriers
Bronchoalveolar lavage	Culture, PCR, (MALDI-TOF)	Calves	Respiratory disease diagnostics
Joint fluid	Culture, PCR	All age groups	Arthritis diagnostics
Quarter milk	PCR, culture	Cows	Mastitis diagnostics
Bulk tank milk	PCR, culture	Cows, herd level	Mastitis diagnostics, detecting carriers
Bulk tank milk	Antibody ELISA	Herd level	Surveillance (mastitis diagnostics)
Serum	Antibody ELISA	All age groups, herd level	Surveillance
Autopsy	Culture, PCR, IHC	All age groups	Different disease manifestations such as pneumonia, arthritis, otitis media, abortion
Conjunctival swabs	PCR, culture	Calves, cows	Detecting carriers
Vaginal swabs	PCR, culture	Heifers and cows	Detecting carriers

Swabs for bacteriological sampling and transport are affordable and widely available. The use of swabs with a plastic shaft is recommended, as swabs with wooden sticks or calcium alginate swabs can inhibit mycoplasma growth or the PCR reaction [198]. Swabs can be used for nasal, conjunctival, and vaginal sampling, and samples from external mucosal surfaces are easily obtained. These swabs can provide information on the infection status of the herd, i.e., detect carrier animals [188-190]. In general, it is not recommended to use NS to detect *M. bovis* in calves with acute respiratory disease, because the presence or absence of *M. bovis* in NS of pneumonic animals does not correlate with clinical disease or the occurrence of *M. bovis* in LRT [199,200]. However, Doyle et al. [201] suggested that NS had a similar ability to detect the occurrence of *M. bovis* in LRT compared to transtracheal wash. Transtracheal wash, BAL fluid, or an NP sample taken with a guarded swab from the nasopharynx can be used in BRD diagnostics [201-204]. Post-mortem examination allows the sampling of body sites from which it is otherwise difficult to obtain a sample, such as the middle ear or joints.

2.3.2 Detection of *M. bovis*

Several methods can be used in *M. bovis* diagnostics. They can be divided into culture-based methods, culture-independent methods, and antibody testing. Table 3 lists the advantages and disadvantages of different detection methods.

Table 3. Advantages and disadvantages of different methods to detect *M. bovis*.

	Culture	**PCR**	**Antibody ELISA**
Sample type	All clinical samples	All clinical and subclinical samples	Blood, BTM, composite milk
Detects	Viable bacteria	DNA	Antibodies
Costs	€€	€€–€€€	€
Time	1 week	1 to 2 days	1 day
Advantages	• Isolate obtained for further studies • Evidence of live bacteria in the sample	• Rapid results • Quantification possible (rtPCR) • Possibility to pool samples • Not affected by antibiotic treatment	• Shedding of M. bovis not necessary • Shows previous infection/exposure • Rapid results
Disadvantages	• Shedding of *M. bovis* necessary • May be affected by antibiotic treatment • Overgrowth of other bacteria possible • Special culture media and some expertise necessary • Species identification needed	• Shedding of *M. bovis* necessary • No isolate obtained • Can detect non-viable bacteria	• Cannot differentiate between previous and present infection • No isolate obtained • Antigen needs to be expressed by all strains and to be immunogenic

2.3.2.1 Culture-based detection methods

Sample storage is an important factor for the successful culture of *M. bovis* from clinical samples. Appropriate collection and storage reduces the potential overgrowth of other bacteria and maximizes mycoplasma growth. It is recommended to store samples at 4 ºC, to keep the samples moist, and culture them within 24 hours [198]. If delayed culture is foreseen, the samples can be sent to the laboratory in mycoplasma broth kept at 4 ºC. If samples cannot be cultured within 2 days, they can be frozen. Freezing has been shown to cause a reduction of 1–2 $\log_{10}$ in the number of viable *M. bovis* in milk [163,205,206]. Mycoplasmas have a high nutritional demand because of their inability to synthesize amino acids and some fatty acids. Mycoplasma culture media should contain yeast extract, tryptone (amino acid sources), serum (sterol source), glucose and/or pyruvate (energy source), antibiotics to prevent the growth of other bacteria, and phenol red or another pH indicator for the detection of mycoplasma growth [198]. Cultures are incubated for 7 to 10 days at 37 °C in 5% CO_2. On solid culture media, *M. bovis* colonies have a typical "fried egg" appearance. The identification of mycoplasma species from cultures requires the use of a post-culture identification test, e.g., species-specific PCR. The limit of detection of *M. bovis* using culture has been estimated to be around 10^2 CFU/mL in milk or BAL [189,207]. The advantages and disadvantages of mycoplasma culture are presented in Table 3.

Another culture-based mycoplasma detection method is the use of MALDI-TOF [208]. The direct transfer method, in which some bacterial mass is transferred from a colony to a detection plate, is not reliable for mycoplasmas due to their very small colony size and growth into agar [208,209]. Bokma et al. [210] have investigated the use of MALDI-TOF to detect *M. bovis* directly and rapidly from BAL samples. They cultured the samples from 24 to 72 hrs in mycoplasma broth with meropenem and vancomycin to suppress the growth of other bacteria. Most of the positive samples were detected after 48 hr of culture. The Se and Sp of the method was 86%, as determined by Bayesian latent class analysis.

2.3.2.2 Culture-independent methods

The most common culture-independent method for detecting *M. bovis* in various sample types is PCR. Both conventional and later rtPCR methods have been published. However, rtPCR has a faster turnaround time and some quantitation is possible. Some published PCR methods are based on 16S rRNA sequences. These sequences only differ in 8 nucleotide positions between *M. bovis* and *M. agalactiae,* hence making the development of 16S RNA-based methods very difficult [207,211]. One study compared the performance of conventional PCR methods in different laboratories [212]. Table 4 lists some rtPCR methods that have been developed to detect *M. bovis* in different clinical samples. Methods based on the sequence of the DNA repair gene *uvrC* are the most common. This

gene has been shown to be highly conserved in *M. bovis* [213]. Some multiplex rtPCRs that detect several bovine Mycoplasma species in the same run have been published [214-217]. Several commercial rtPCR kits are available to detect *M. bovis* in mastitis milk and other types of bovine samples.

Table 4. Published rtPCR methods to detect only *M. bovis* in various sample types.

Target gene	Sample	Limit of detection	Reference
16S rRNA	Milk, lung	Milk: 550 cfu/ml milk; lung 650 cfu/25 mg	[218]
oppD	Milk, nasal swab	Milk: 272 cfu/ml	[189]
uvrC	Milk	Milk sediment: $2x10^3$ cfu/ml	[219]
uvrC	Milk, nasal swabs, joint fluid, semen	83 copies of *uvrC* gene in a PCR reaction	[220]
uvrC	Milk, semen, swabs	Milk: 130 cfu/ml; semen: $1.3x10^5$ cfu/ml; swab: $1.3x10^6$ cfu/ml	[215]
uvrC	Milk, lung	40 copies of *uvrC* gene in a PCR reaction	[221]

Wisselink et al. [207] reported results from an interlaboratory trial of six laboratories that used five different DNA extraction methods, seven different real-time and/or end-point PCRs targeting four different genes, and six different rtPCR platforms. Despite the multiplicity of methods used, the study revealed comparable diagnostic performances for all of the tested PCR methods. Weak positive results (Ct values between 37 to 40) were obtained from some non-target Mycoplasma species. This highlights the importance of thorough assessment of the cut-off value during the validation of rtPCR tests in individual laboratories.

Isothermal amplification methods to detect *M. bovis* in mastitis milk or NS/BAL samples have been published. In particular, the recombinase polymerase amplification assay (RPA) can be performed at a low temperature (about 37 °C) and only takes 20 to 30 min to perform. Hence, it could be used as a point-of-care test in the future. Several RPA assays using either the lateral flow stick method to detect the PCR product or real-time RPA have been published [222-224]. The limit of detection of these assays is from 10 to 40 copies per reaction. Loop-mediated isothermal amplification (LAMP) is performed at higher temperatures than RPA (over 60 °C) and takes 30 to 60 min to perform. The result of a LAMP reaction can be detected visually. LAMP methods have been developed to detect *M. bovis* in respiratory samples [225] and different types of milk samples [226,227].

Other published culture-independent diagnostic methods include the use of DNA microarrays [228] and nanopore sequencing [229].

2.3.2.3 Serological diagnosis using an antibody ELISA

While successful culture or PCR detection relies on the presence of *M. bovis* in the sample, the purpose of an antibody test is to detect animals that have been exposed to the pathogen and have had time to develop a humoral immune response. Anti-*M. bovis* antibodies in serum and milk are usually detected using an indirect ELISA test. Several studies reporting results from a vaccine trial or an experimental challenge have used in-house ELISA tests to evaluate the serological response [157,230-234]. Comparing such results to evaluate, for instance, seroconversion and the longevity of antibodies is very difficult. Commercial ELISA tests are produced in a standardized manner, are quality controlled, and are generally available, making them more suitable for comparative studies.

The commercially available ELISA kits BIO K 302 and BIO K 260 have been provided by Bio-X Diagnostics (Jemelle, Belgium) for quite a long time. The antigen used to coat the plates is the proprietary knowledge of the manufacturer. BIO K 302 was used to investigate the antibody response in cows over a 15-week period in herds that had mastitis, pneumonia, and/or arthritis symptoms and in which *M. bovis* had been confirmed in some samples. Both serum and composite milk samples were analyzed. The principal findings were that after an outbreak, seroconversion occurred early but showed a high level of variation between individual cows. Thus, serology does not appear to be suitable for individual animal testing, but it might be useful for detecting herd-level exposure to *M. bovis*. In this study, the individual antibody response in composite milk was only suitable for detecting cows that had had mastitis [235]. An Australian study using the same ELISA revealed that almost all studied cows (15/16) confirmed to have *M. bovis* mastitis 7–13 days prior to blood sampling had seroconverted [190]. From four Australian herds in which an *M. bovis* mastitis outbreak had occurred 40 to 68 days before blood sampling, a total of 200 serum samples from randomly selected cows from the main milking herds were tested. The mean percentage of seropositive cows in the four herds was 76%, 40%, 20%, and 16%, and was highest in the two herds with the highest numbers of cows with *M. bovis* mastitis [197]. This result again suggested that BIO K 302 could be used in herd-level diagnosis at least up to 2 months after a mastitis outbreak. The antibody response of 83 young calves from the same herds as in study by Petersen et al. [236] was analyzed using BIO K 302 ELISA. Again, there was large variation in the antibody response between calves, and the ELISA test rarely detected antibodies in calves younger than 2 months of age. Although the tested calves either showed signs of *M. bovis* infection or were housed together with diseased calves, they rarely seroconverted. The authors concluded that use of BIO K 302 in calves under 3 months of age cannot be recommended [236]. In a study using sera from Canada, England, and Australia, all from experimental challenge studies, WB and BIO K 302 were compared. A recombinant fragment of VspA was used as the antigen in

WB. The Se for WB and BIO K 302 was 74% and 47%, and Sp was 88% and 96%, respectively [237]. This means that WB was more sensitive, i.e., detected more positive animals than BIO K 302, but was also less specific, meaning that some WB-positive animals were not truly diseased.

Wawegama et al. [238] developed an in-house ELISA based on a recombinant fragment of mycoplasma immunogenic lipase A (MilA). They compared the Se and Sp of the MilA ELISA with BIO K 302 using serum samples from an experimental challenge study. The Se for BIO K 302 and MilA was 37% and 87%, and Sp was 95% and 90%, respectively [239]. The performance of this in-house ELISA was compared with BIO K 302 in the calf study by Petersen et al. [236]. The antibody response to MilA rose above the cut-off value in nearly all calves, and antibodies were detected from 3 weeks of age onwards, thus proving that the MilA ELISA was very sensitive compared to BIO K 302 in a field study and can be used in young calves.

The applicability of BTM antibodies for *M. bovis* diagnosis using BIO K 302 has been assessed in two studies [240,241]. In a Danish study, the main findings were that the BTM antibody level increased together with an increase in the number of antibody-positive cows among lactating cows, but the level of serum antibodies in young stock did not correlate with the BTM antibody level, and a high antibody level in BTM appears to be short-lived [240]. Parker et al. [241] found that in Australia, the BTM antibody level was mostly associated with the time since the start of an outbreak and it appeared that BIO K 302 BTM antibody measurement was quite reliable in predicting *M. bovis* exposure up to eight months after the outbreak. The within-herd seroprevalence explained little of the variation seen in the BTM antibody level. In conclusion, the use of BTM antibodies in herd-level *M. bovis* diagnosis is not without problems, and further studies on antibody dynamics and longevity are warranted.

Table 5. Results from different serum antibody studies using BioX, MilA or WB to detect anti-*M. bovis* antibodies in serum.

ELISA	Study population	Se/Sp	Prevalence or main findings	Reference
Bio K 162	361 herds, 3670 animals	NA	Herd prevalence 80.9%, animal 76.7%	[114]
In-house MilA, BIO K 302, BIO K 260	30 experimentally infected, 5 controls, paired sera	MilA Se 87%, Sp 90.0%; K 302 Se 37%, Sp 95%; K 260 Se 13%, Sp 100%		[239]
In-house MilA	7448 feedlot cattle	Se 94.3%, Sp 94.4%	At cut-off 105 AU, 13.1% seropositive on entry to the feedlot; 6 weeks later, 73.5% seropositive	[239]
BIO K 302	4 herds, 120 cows, herds with confirmed Mbad	NA	Serum antibody levels highly variable between individual cows; antibodies only in the milk of those cows that had had mastitis; ELISA testing only suitable for herd-level diagnosis	[235]
In-house MilA, Bio K 302	4 herds, 83 calves	NA	MilA (at cut-off 135 AU) detected antibodies in calves from three weeks of age onwards; the use of BIO K 302 in calves was not recommended, as even diseased calves rarely seroconverted	[236]
BIO K 302	118 breeding bulls	NA	Prebreeding seropositive 9%, post-breeding 46%, no clinical signs	[242]
BIO K 302	16 cows, mastitis confirmed 7–13 days earlier	NA	15 out of 16 cows seropositive	[190]
WB, BIO K 302, BIO K 260	Experimental challenge studies, Canada n = 10, UK n = 5, Australia n = 35	WB Se 74%, Sp 88; K 302 Se 47%, Sp 96%; K 260 Se 28%, Sp 100%	WB antigen recombinant VspA fragment	[237]
BIO K 302	4 dairy herds with Mb mastitis, 50 samples per herd	NA	Seroprevalence within herd from 16% to 76%; highest in herds with the highest number of clinical Mb mastitis	[197]

Table 6. Results from different studies using BioX BIO K302 ELISA to detect anti-*M. bovis* antibodies in BTM.

ELISA	Study population	Se/Sp	Prevalence or main findings	Reference
BIO K 302	3437 dairy herds	Se 60.4%, Sp 97.3% (cut-off 37 ODC%), Se 43.5%, Sp 99.6% (cut-off 50 ODC%)	The authors recommended the use of an ODC% cut-off value of 50 to increase specificity, Bayesian latent class analysis	[106]
BIO K 302	31 herds with Mbad, 8 control herds	NA	Increasing prevalence of milk antibody-positive cows was the only variable associated with increasing BTM antibodies. Seroprevalence in young stock did not correlate with BTM antibodies. BTM antibodies can detect some infected herds, but only those in which there is a high enough prevalence of Mb mastitis	[240]
BIO K 302	19 previously Mb-exposed dairy herds and 6 control herds; 192 BTM samples and 50 blood samples per herd from cows contributing milk to the tank	NA	The BTM ELISA antibody level correlated with time since the start of the mastitis outbreak and was above the cut-off ODC% of 37 eight months after the outbreak. With-in herd serum seroprevalence explained little of the variation in BTM ELISA ODC%	[241]
BIO K 302	120 dairy herds with >100 cows	NA	Herd prevalence 48.3% (95% CI 39.1; 57.6)	[109]

2.3.3 Molecular epidemiology

Several molecular microbiological tools have been used in the past to study the genetic structure of *M. bovis* populations (usually within a country) and to trace transmission routes, as well as to determine whether clones linked to special disease outcomes such as mastitis or pneumonia exist. These methods have mostly been based on DNA fragment fingerprints: random amplified polymorphic DNA [184,243], amplified fragment length polymorphism [74,243,244], pulsed-field gel electrophoresis ([77,244-248], and multiple locus variable number of tandem repeats analysis [184,249-252]. Additionally, insertion sequence typing has been used in some studies [105,253,254].

These methods have now largely been replaced by DNA sequencing-based analyses. MLST is based on the sequencing of a few, usually seven, well-conserved loci. Each novel sequence variant at each locus is given a number in order of discovery, and the numbers together form an allelic profile (e.g., 3–5–2–4–6–9–2), which is then assigned an arbitrary sequence type (ST) number [255]. The advantage of MLST is that sequence data are unambiguous, electronically portable, and the central curated database PubMLST (https://pubmlst.org) holds the reference allele sequences and lists of STs for individual organisms. Two MLST typing schemes for *M. bovis* based on seven completely different loci were proposed at the same time [256,257]. The scheme proposed by Register et al. [257] became the curated one in PubMLST. This scheme proved to be informative and discriminative and showed how international cattle trade has led to the expansion of certain STs into many countries and continents [248,258,259]. However, it soon became evident that some *M. bovis* strains did not possess the *adh-1* gene, one of the seven genes used in the MLST scheme [260,261]. Register et al. [262] found that the *adh-1* locus was absent in 1.4% of isolates obtained prior to 2011, compared to 14.2% of isolates originating after that time. This locus in the scheme was replaced by the *dnaA* locus. The discrimination index of the new scheme is 0.914 and it differentiated 88 STs among 448 isolates analyzed [262]. Of note, *M. bovis* strains circulating in Denmark, Sweden, and Finland after 2010 were all found to belong to ST29. This ST was also detected in mastitis isolates from Israel from the years 2012 to 2017 and one pneumonic isolate from Hungary from 2016 [263].

The development of next generation sequencing methods has reduced the cost of sequencing whole genomes, as well as the time needed for this. One of the biggest challenges is how to analyze the vast raw read data in a fit-for-purpose manner. Basically, in the epidemiological and phylogenomic analysis of bacteria, three approaches are used: wgSNV, cgMLST, and wgMLST. wgSNV compares single nucleotide differences between isolates either in comparison to a reference genome or utilizing bioinformatic tools without the need for a reference genome. This approach can give high resolution, but the drawback is the low comparability between different studies (i.e., due to different SNV threshold

settings and different reference genomes used) [264]. Cg/wgMLST methods assess the allele diversity of all the genes universally found in a particular genus using gene-by-gene comparison. The problem with these methods is how to define the core and accessory genome of a species and how to make these allele schemes internationally accepted to be able to have a central curated database [264]. No publicly available cg/wgMLST scheme yet exists for *M. bovis*, but *ad hoc* schemes have been used [164,265].

WgSNV analysis to infer genetic diversity and phylogenomics in different *M. bovis* populations has been used in several studies [263,265-269]. In the studies of Parker et al. [266] and Bokma et al. [267], the genome of *M. bovis* PG45 was used as a reference genome, whereas other studies used the kSNP tool [270] without genome alignment on a reference genome. Australian strains were found to be very homogeneous, with 50 SNV at maximum between any two isolates, and no body site/disease-specific isolates could be found [266]. In contrast, Belgian isolates were divided into five clusters and SNV ranged from 60 to 1512 within a single cluster. Globally, Belgian isolates clustered with Israeli, European, and North American isolates. No genotypically specific strains were found within the beef, dairy, or veal calf sector [267]. Yair et al. [269] demonstrated that wgSNV analysis clustered the isolates included in their study based on geographical origin: strains from Europe clustered together and separately from Chinese and Australian strains. Israel imports thousands of calves every year from European countries and Australia, so it was not a surprise that these Israeli isolates were found in both clusters. Interestingly, a dominant genotype among Israeli mastitis isolates was seen. Kumar et al. [268] conducted a large-scale comparative analysis of 250 *M. bovis* genomes, including 70 isolates from bisons. They confirmed the results of Yair et al. [269], showing that Australian strains formed their own clade together with Chinese and some Israeli strains. USA isolates displayed high genetic variation and were dispersed into five different clades. Four of these were only occupied by the USA isolates and the fifth contained Canadian and USA isolates and one isolate each from Switzerland, Israel, and Lithuania. There was no clear-cut clustering based on host origin (cattle or bison). Tardy et al. [263] examined a collection of strains from the 1980s and 1990s and contemporary strains mainly from Denmark and found that a new clonal type had emerged in Denmark at some time after 2008, which has since 2011 been the dominant type. The same clone has spread to Sweden and Finland and was also seen to some extent among isolates from the Netherlands, but not in France.

Ad hoc cgMLST was compared with cgSNV analysis using a collection of 129 Canadian isolates from feedlots. These two methods provided similar phylogenetic resolution and produced similar topology of the strains when the results were visualized using minimum spanning and neighbor-joining tree methods. However, the highest resolution was provided by the wgSNV method, and this approach was less sensitive than cgMLST and cgSNV to poor-quality sequence data [265].

Interestingly, in the study of Tardy et al. [263], cgMLST (500 genes in that study) provided better resolution than wgSNV analysis, but the reason for this was not discussed.

Kumar et al. [268] also conducted core and pangenome analysis across 250 isolates and found 283 conserved and a pangenome of 1186 coding sequences, respectively. *M. bovis* appears to have an open pangenome that will grow with the addition of new genomes to databases. McInerney et al. [271] demonstrated that genes in the accessory genome most likely aid bacteria in adapting to different niches, so in the future, analysis of the *M. bovis* accessory genome might provide valuable information on how this species, for example, occupies different body sites. Another interesting finding in the study by Kumar et al. [268] was that the number of *vsp* genes appeared to be reduced in many isolates from the 13 genes seen in PG45, and some were highly distinct at the sequence level compared to PG45. This warrants further sequence-level comparisons of *vsp* genes, as these differences might affect the adhesion properties of these strains, as well as the usefulness of Vsps in anti- *M. bovis* antibody studies.

2.4 Control of *M. bovis*

2.4.1 Vaccination

Several research groups have developed experimental vaccines against *M. bovis* respiratory disease without much success (reviewed in [5,272]). The vaccine types tested have either been bacterin vaccines in which the mycoplasma cells have been inactivated with formalin or saponin [157,230,273-275] or subunit vaccines containing different recombinant proteins deemed to be immunogenic, as well as different membrane fractions [231-234]. These vaccines have been administered subcutaneously. In one study, several passages of the investigated *M. bovis* strain led to attenuation in virulence, and this attenuated strain was used as a live vaccine intranasally [276]. Most of the tested vaccines have been found to produce a good antibody response in young calves, but vaccinated animals are not significantly protected against *M. bovis* challenge, and in some studies, the vaccine appeared to exacerbate the disease. Commercial bacterin vaccines are licensed in the USA. These have been studied in two field trials including dairy calves vaccinated at the age of 3 days and boosted 11 days later [277] and veal calves vaccinated at the age of 4 weeks [278]. Both studies concluded that none of the commercial vaccines was efficacious in preventing *M. bovis*-associated disease, despite increased serum antibody titers, and in one dairy herd, significantly more otitis media was recorded in vaccinated calves. Due to the increased respiratory disease burden caused by *M. bovis* in British cattle herds, a commercial bacterin vaccine was temporarily given a license into the UK and is now being evaluated in the field [62].

There are many challenges in *M. bovis* vaccine development. One is the lack of a challenge model that would consistently reproduce the respiratory disease and produce it in the magnitude seen in field cases. Kanci et al. [158] described the reproduction of *M. bovis*-associated respiratory disease in calves using an aerosolized mycoplasma culture. This method could be applicable in the future in vaccine testing. It is known that the immune response to *M. bovis* in calves is skewed to the Th2 response and IgG1 production, but a successful vaccine probably also needs to induce a Th1 response to aid in shaping the antibody response towards IgG2 [5]. Because *M. bovis* is not the only player in the BRD complex, it should ideally be included in a multivalent BRD vaccine. Several *M. bovis* complete genomes have been published, but a detailed comparative analysis to increase our understanding of the genetic and antigenic variability among different strains is lacking. These analyses could pave the way to applying a systems vaccinology approach to construct an efficient vaccine [279].

2.4.2 Treatment and antibiotic resistance

Until effective vaccines are available, biosecurity, sanitary preventive measures, and antibiotic treatment are the methods to control *M. bovis* infections. Mycoplasmas lack a cell wall and cannot synthesize folic acid, making them intrinsically resistant to β-lactam antibiotics, glycopeptides, and sulfonamides/trimethoprim. They are also naturally resistant to polymyxins, rifampicin, and quinolones such as nalidix acid [280]. The most widely used antibiotics to treat *M. bovis* infections are tetracyclines, macrolides, tilmicosin, florfenicol, and fluoroquinolones [280,281]. In general, antibiotic treatment of *M. bovis* infections is unrewarding. First, control of the infection requires the early detection of *M. bovis*, and treatment needs to be started early to prevent inflammatory tissue changes such as caseonecrotic foci in the lungs. If this happens, it will further decrease the effect of antibiotics [63]. Secondly, antibiotic resistance in contemporary *M. bovis* isolates is growing [263,281-287]. Having stated this, no standard exists for the *in vitro* determination of antibiotic activity against veterinary mycoplasmas, and neither have breakpoints been defined. The International Research Programme on Comparative Mycoplasmalogy has proposed recommendations for antimicrobial susceptibility testing of animal mycoplasmas [288]. However, variations from the recommended method have been reported, making it difficult to compare the results from different studies [281]. Examining the report of Klein et al. [287], in which isolates from 2012 to 2016 from France, Italy, Hungary, Great Britain, and Spain were examined, MIC_{90} values (mg/L) were as follows: oxytetracycline 32, florfenicol 8, enrofloxacin 8, danofloxacin 1, tylosin, tilmicosin, tulathromycin, and gamithromycin all >64. Isolates from France, Great Britain, and Hungary had MIC_{90} values for enrofloxacin of 0.5 to 1 mg/L, whereas strains from Italy and Spain showed MIC_{90} values of 16 and 32 mg/L, respectively. Based on CLSI

clinical breakpoints for bovine respiratory pathogens, this means that no effective antibiotics against *M. bovis* are available in Italy and Spain, and in other countries, the only option seems to be fluoroquinolones. However, fluoroquinolones belong to highly critical and important antibiotics in human medicine, and their use in veterinary medicine should only be as a last resort after sensitivity testing has been conducted, and when no other antibiotic would be clinically effective [289]. Godinho [290] suggested that a high MIC for tulathromycin (>64 mg/L) does not necessarily mean a lack of clinical efficacy, as a high MIC strain was effectively treated in a clinical trial. The contemporary clone in Nordic countries is resistant to oxytetracycline and tilmicosin, intermediate to resistant to florfenicol, and sensitive to enrofloxacin [263]. The first-line treatment recommendation in Finland for BRD is oxytetracycline and the second line is benzylpenicillin or macrolides [291].

Mycoplasmas do not harbor plasmids. The resistance mechanisms described in *M. bovis* are point mutations in their chromosome, leading to modification of the binding site of the antibiotic. Resistance to tetracyclines and spectinomycin is associated with mutations in 16S rRNA. Mutations in 23S rRNA and proteins L4/L22 are associated with macrolide, florfenicol, and lincosamide resistance. Fluoroquinolone resistance is caused by mutations in DNA gyrase and/or in DNA topoisomerase IV [259,280,292-299].

2.4.3 Eradication

Once *M. bovis* has established itself in a multi-age cattle farm, eradication can be difficult [300]. Two studies have described eradication in a single dairy herd suffering from *M. bovis* mastitis. The eradication was based on the efficient identification and culling of infected cows [301,302]. Respiratory disease and arthritis occurred in calves, but no control measures applied to young stock were described ([302]. In contrast, Brys et al. [303] described how *M. bovis*-free raising of animals is possible in a dairy farm with *M. bovis*-associated disease. This method involved the transfer of newborn female calves immediately after birth to an isolated calf barn and keeping them separate from other cattle until they calved. Intensive NS and antibody testing over a 30-month period did not show evidence of *M. bovis* infection in these animals. Some Finnish dairy farms have successfully applied this method combined with culling of all *M. bovis* mastitis cows to achieve a low-risk status [304]. In Ireland, the Teagasc Research Centre herd had to be depopulated due to an animal testing positive for bovine spongiform encephalopathy. When the herd was restocked, only animals that were negative in anti-*M. bovis* ELISA testing were purchased. The herd's *M. bovis*-free status had been held for at least two years after restocking [305].

New Zealand (NZ) was free of *M. bovis* until July 2017, when an outbreak occurred in a dairy herd. A year after this, NZ decided to attempt national

eradication. This was based on extensive testing of BTM samples for antibodies, and in the case of a positive finding, further testing of individual cow serum samples for antibodies, as well as serological testing of fattening cattle at three stages [306]. Positive premises are depopulated, cleaned, and restocked after a pause in production for a couple of months. As of April 2022, altogether 272 premises had tested positive and 270 had already been declared free of infection [307]. WGS of several hundred isolates has demonstrated that one type or at maximum three very closely related strains had entered the country either at the end of 2015 or the beginning of 2016. This also supports the idea that eradication is feasible.

2.4.4 Controlling the spread of mycoplasmas through bovine semen trade

The number of frozen and liquid cattle semen doses sold globally for AI is over 232 million and 11 million, respectively [308]. Theoretically, semen from one *M. bovis* infected bull could result in the production of numerous contaminated semen straws. *M. bovis* has been shown to remain alive in semen straws stored in liquid nitrogen for years [42]. Experimental inoculation studies have demonstrated the infectivity of *M. bovis* in the female reproductive tract [95,309,310]. Transmission of *Campylobacter fetus,* leptospires, and mycoplasmas through semen trade is controlled by EU legislation (implementing act 2020/686) and the OIE Terrestrial Code (chapter 4.7.7), which state that antibiotics or mixtures of antibiotics that are effective against the above-mentioned bacteria must be added to semen or contained in semen diluents. The mixtures of antibiotics mentioned in EU legislation and the OIE Code are gentamicin (250 µg), tylosin (50 µg), lincomycin-spectinomycin (150/300 µg), penicillin (500 IU), streptomycin (500 µg), lincomycin-spectinomycin (150/300 µg), amikacin (75 µg), and divekacin (25 µg). The GTLS mixture was originally developed and efficacy tested against bacteria, including *M. bovis*, by Shin et al. [311]. However, in their studies, the concentration of each antibiotic was double the concentration mentioned in the EU implementing act and OIE Code. CSS in the USA has adopted the method described by Shin and colleagues. Visser et al. [312] have argued that the GTLS mixture according to Shin is at best mycoplasmastatic and is not capable of totally eliminating *M. bovis*. Increased resistance to several antibiotics in contemporary *M. bovis* strains can further complicate the use of antibiotics in semen extender to control *M. bovis* transmission.

2.4.5 Finnish *M. bovis* control program

The Finnish *M. bovis* control program was established in 2013 and is administered by the Naseva register [313]. The costs of sampling, laboratory testing, and herd

health visits are financed by farmers. The main aim of the program is to reduce the risk of transmitting infection to dairy and suckler cow herds through animal trade. This further improves animal welfare and reduces the use of antibiotics in calf-rearing units. The key elements of the program are regular testing of QMS samples from mastitis cases using PCR including *M. bovis*, the monitoring and sampling of animals showing suspected clinical signs, nasal swab sampling of calves, and the control of animal trade. Meat inspection data are also followed, because a study by Haapala et al. [314] demonstrated that lung lesions are more common in infected herds. The program is described in Table 7. The herds at the Naseva national level should be free of *M. bovis*. Infected herds obtain financial support to pay for testing. They reach the national level after three negative samplings of NS from calves taken at 4- to 8-month intervals and after having negative results from regular QMS and BTM sampling. Herds at the national level can join the voluntary control program. The herds in the control program are categorized into level B (joining level) and the highest level A. The *M. bovis* status of the herd is documented in the Naseva register and this information is available to authorized users. The use of health certificates is obligatory when purchasing cattle or attending cattle shows.

Table 7. Description of the voluntary Finnish *M. bovis* control program

Requirements	Classification of herds in the Naseva Register			
			M. bovis control program	
	M. bovis-infected herds during control measures	Naseva national-level herds	Joining-level (B-level) herds	A-level herds
Herd health				
Veterinary herd health visits	Minimum 2/year	Minimum 1/year	Minimum 2/year	Minimum 2/year
Veterinary monitoring of herd health and meat inspection data in the Naseva Register	Yes	Yes	Yes	Yes
Health care and biosecurity plan	Yes	Yes	Yes	Yes
Medication data documented in Naseva	Voluntary	Voluntary	Mandatory	Mandatory
Risk assessment or use of Biocheck.UGent®	Yes	Voluntary	Yes	Yes
Presence of *M. bovis* infections	Yes	No	No	No
Sampling for Mycoplasma bovis				
Sampling of healthy calves for *M. bovis* (PCR or Elisa)[a]	Three sampling occasions with negative results to reach national level[b]	No	Twice[b]	2/year (dairy), 1/year (suckler cows)
Testing of BTM for *M. bovis* by PCR	Yes[c]	No	Twice[b] (dairy)	2/year (dairy)
Routine testing of QMS for mastitis pathogens (by PCR including *M. bovis*)	Yes	Recommended	Yes	Yes
Sampling of clinical cases	Yes	Yes	Yes	Yes
Control of cattle movements				
Movements of cattle from the herd	Only to infected calf rearing or finishing units and slaughter	Yes	Yes	Yes
Use of health certificates in cattle trade	Recommended	Recommended	Mandatory	Mandatory
Regular testing of mastitis QMS and BRD	Recommended	Recommended	Mandatory	Mandatory
Screening the herd of origin for symptoms of *M. bovis*	Recommended	Recommended	Mandatory	Mandatory
Participation in cattle shows	Not allowed	Not recommended	Only shows for A-level herds	Only shows for A-level herds

[a]Nasal swab sampling of all (max 20) calves aged 1 week to 6 months; in herds with less than 10 calves, additional antibody testing of 15 animals aged over 3 months

[b]At 4–8-month intervals

3 AIMS OF THE STUDY

The general aim of this thesis study was to gain knowledge of the characteristics of *M. bovis* infections in Finnish dairy farms, to investigate a possible new transmission route of *M. bovis* infection, and to evaluate methods that can be used to control *M. bovis* infections. The specific objectives were as follows:

1. To describe and improve understanding of the characteristics of *M. bovis* infection on Finnish dairy farms;
2. To investigate the dynamics of the antibody response in young stock and cows on dairy farms recently infected with *M. bovis* using two different ELISA tests;
3. To evaluate the use of BTM serology and PCR to detect *M. bovis* infection;
4. To investigate the possible role of contaminated commercial semen as a route of transmission of *M. bovis* into naive dairy herds;
5. To assess the efficacy of different antibiotics in destroying *M. bovis* in commercial bovine semen production;
6. To evaluate the suitability of NS and NP sampling of young calves in the *M. bovis* control program;
7. To determine the most cost-efficient way to detect *M. bovis* in acute BRD;
8. To evaluate different methods to isolate *M. bovis* DNA from extended bovine semen.

4 MATERIALS AND METHODS

4.1 Study herds and grouping of the farms A to S according to their infection status (I, II, III)

Animal Health ETT has maintained a voluntary *M. bovis* control program in Finland since 2013. All dairy herds found to be infected with *M. bovis* during 2013 to 2016 were encouraged to participate in the control program and the research project "Mycoplasma bovis in dairy herds". Study I included 19 recently infected dairy herds (herds A to S in study I, herds 1 to 19 in study III). Infection with *M. bovis* had not previously been detected in any of the herds, despite continuous mastitis pathogen testing, including real time PCR for the detection of *M. bovis*.

The farms were advised to cull cows with mastitis caused by *M. bovis*, to avoid purchasing new animals, to house calves separately from the cows, not to feed mastitis milk to calves, and to follow appropriate hygiene measures.

To analyze the seroprevalence and dynamics of herd *M. bovis* antibodies in study I, the study herds were classified into six infection status groups based on the detection *of M. bovis* at each visit. A sampling visit was considered positive if *M. bovis* was detected in the herd by real-time PCR or the culture of NP or NS swabs, or in clinical, post-mortem, or mastitis samples, with the status at each visit classified as positive or negative.

In study II, herd data from farms X and Y were collected using the centralized health care register Naseva [313] and using a questionnaire. Insemination data (dates, bulls, and lots) were gathered from the Finnish Animal Breeding Association (FABA). The distance to the closest cattle farm was determined from a national register.

4.2 Sampling scheme in the study herds (I, III)

Veterinarians visited each of the farms A to S (I), which were the same as farms 1–19 in study III, four times, at approximately 6-month intervals. Two more dairy herds (herds 20 and 21, study III) were sampled twice with a six-month interval, and nine herds (herds 22 to 30, study III) were sampled once. During each visit, the veterinarian collected NP and NS swabs from calves between one week and nine months of age. A total of 5 NP and 10 to 20 NS swabs were collected at each visit, depending on the number of calves on the farm. In herds with a sufficient number of young stock, 15 serum samples were collected from each age group of the young stock (3–6, 6–9, and 9–12 months of age) and from cows, with a maximum of 65

blood samples collected per herd (I). Farmers were advised to carefully monitor cattle for mastitis and other clinical signs and to submit QMS samples from all cases of subclinical and clinical mastitis for real-time PCR testing, as well as monthly BTM samples for rtPCR and ELISA antibody testing. As part of the study, the farms could send other clinical samples, including calves with a disease suspected to have been caused by *M. bovis*, for post-mortem examination (I).

Nasal swabs (Transystems, Copan, Brescia, Italy) were taken prior to NP (I, III) and BAL (only in III). The nostrils were cleaned with a paper towel and the swab was inserted into a nostril to a depth of ~13 cm. Two nasal swabs, one for PCR and one for mycoplasma culture, were simultaneously collected from calves with acute respiratory disease (III) and one NS was taken from healthy calves in dairy herds (I, III). NP swabs were taken with 27-cm-long guarded swabs (Medical Wire Equipment Ltd, Corsham, England). The sheathed swab was inserted into the ventral nasal cavity approximately one centimeter rostral to the medial canthus of the eye, and the swab was advanced a few centimeters to the nasopharynx area and rotated. The swab was withdrawn into the sheath before removal. BAL samples were collected using a self-made sterile guarded plastic catheter inserted through the nose into the trachea. The inner catheter was pushed out and advanced until it wedged in a bronchus. Thirty to forty milliliters of sterile prewarmed 0.9% saline was injected and immediately aspirated back into the syringe. The NP swabs intended for mycoplasma culture were soaked in D broth [315], and 0.5 ml of the BAL sample was transferred into D broth. The samples were transported to the laboratory within 24 hours in styrofoam boxes with a freezer pack.

4.3 Mycoplasma culture and DNA extraction from broth cultures, NS, and semen samples (I–IV)

Samples in D broth were diluted tenfold up to 10^{-2} in F broth [316] in tightly closed tubes. The broths were incubated at 37 °C for 3–5 days, and growth and color change were monitored every second day. All broth cultures were examined for the presence of *M. bovis* by rtPCR, and suspected positive cultures were sub-cultured onto F medium plates [316]. The plates were incubated in 5% CO2 at 37 °C for seven days and inspected every second day under the microscope for mycoplasma colonies.

DNA was extracted from broth cultures as follows: 200 µl of the culture broth was boiled for 5 min followed by centrifugation at 12,000 g for one minute. The supernatant was used as a template in rtPCR. DNA extraction from NS was performed according to Sachse et al. [189]. In study II, DNA was extracted from semen straws using the QIAamp Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for blood and body fluids. Three different protocols

to extract DNA from spiked semen were compared in study III. In each method, 200 µl of semen was used as starting material. Method 1 was automated DNA extraction using a QIAcube robotic workstation (Qiagen, Hilden, Germany) and the blood and body fluids protocol with the QIAamp DNA mini kit. The elution volume was 150 µl. In method 2, 200 µl of semen was combined with 200 µl of 2% Triton-X 100 (Sigma Aldrich) in 10 mM Tris – 1 mM EDTA (pH 8) buffer. The sample was thoroughly vortexed and pelleted at 13,000 g for 5 min. DNA was extracted from the pellet using the QIAcube robotic workstation and the bacterial protocol with the QIAamp DNA mini kit. The elution volume was 150 µl. Method 3 was modified from the OIE Terrestrial Manual method to isolate DNA from bovine semen for herpesvirus PCR (chapter 3.4.11, adopted May 2017). In method 3, 200 µl of semen was centrifuged at 13,000 g for 10 min and the supernatant was discarded. The pellet was mixed with 200 µl of InstaGene™ matrix (Bio-Rad, Helsinki, Finland), 5.8 µl of proteinase K (20 mg/ml), and 7.5 µl of DL-dithiothreitol (1 M). Samples were incubated at 56 °C for 30 minutes and then vortexed at high speed for 10 seconds. The tubes were boiled in a water bath (100 °C) for 8 minutes and then vortexed at high speed for 10 seconds. Then, the tubes were centrifuged at 10,000 g for 3 minutes. The supernatant was transferred into a new microtube and stored at -20 °C.

4.4 *M. bovis* rtPCR (I–IV)

An rtPCR assay targeting the *oppD* gene of *M. bovis* as described by Sachse et al. [189] with minor modifications was used throughout all studies to detect *M. bovis*. Minor modifications included the use of pUC19 as an internal amplification control, as described in Fricker et al. [317]. BHQ1 was used instead of TAMRA in the pUC19 probe. rtPCR reactions were run using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA).

QMS and BTM samples were analyzed by private laboratories using a commercial rtPCR assay for 16 mastitis pathogens, including *M. bovis* (Pathoproof® Complete 16-kit, Thermo Fisher Scientific, Finland), according to the manufacturer's instructions.

4.5 Detection of *M. bovis*-specific antibodies (I)

Two ELISAs were used to detect *M. bovis*-specific antibodies, the Bio K260 (Bio-X Diagnostics, Jemelle, Belgium) commercial ELISA and the in-house MilA ELISA [238]. The Bio K260 ELISA was performed according to the manufacturer's instructions. The MilA ELISA was performed as described previously [238], with the concentration of *M. bovis*-specific antibodies in each sample calculated in

antibody units (AU) by comparison with a series of standards on each plate using an ELISA analysis program (http://www.elisaanalysis.com). A result of >135 AU was interpreted as positive.

BTM samples were analyzed using both ELISA tests. For the Bio K260 ELISA, 1 mL of the BTM was centrifuged at 4000 g for 20 min and 100 µl of middle layer (skim milk) was used in the ELISA. For the in-house MilA ELISA, the BTM samples were diluted 1:20 before analysis. The concentrations of anti-*M. bovis* antibodies in the BTM samples collected at or near the sampling visits (n = 68, study I) were compared with the mean concentration of anti-*M. bovis* antibodies in the serum samples collected from the cows in the herd at the same visit over all four visits.

4.6 WGS and cgMLST

WGS was used to compare the genomes of *M. bovis* isolates from farms X and Y and bull semen, seven isolates obtained from 2012 to 2015 from diseased cattle in Finland, and one isolate from Estonia. WGS was performed at the Danish Technical University, Department for Biotechnology and Biomedicine, Lyngby, Denmark using the Nextera XT kit (Illumina, San Diego, CA, USA) and Illumina 300 bp paired-end sequencing.

cgMLST analysis of the sequencing reads was performed by creating an *ad hoc* cgMLST scheme using Ridom SeqSphere+ (Ridom GmBH, Münster, Germany) software. *De novo* assembly of the reads was carried out using Velvet assembler 1.1.04 in Ridom SeqSphere+ software. Automated k-mer and coverage cut-off optimizations were performed for each assembly. The BLAST-based target definer function [318] of the software was operated to identify 527 core genome and 168 accessory genome target loci from the *M. bovis* reference strain PG45 and seven other complete *M. bovis* query genomes obtained from GenBank. A minimum spanning tree within the software was constructed to visualize the result.

4.7 Experimental inoculation of semen with *M. bovis* and antibiotic treatments (IV)

All studies were conducted in the laboratory of an AI center producing commercial AI straws using industrial-scale procedures. This was possible because the semen production ceased in this center after these experiments. Semen from three bulls was collected into sterile collection tubes at the AI center of Viking Genetics, Hollola, Finland. The motility of each semen batch was evaluated microscopically at 200x magnification using prewarmed glass slides and coverslips. The viability

and concentration of each batch was analyzed using flow cytometry (CyFlow, Partec, Germany). Pooled raw semen (0.3 ml) was cultured in F broth to detect possible mycoplasma contamination. The final sperm cell concentration was 12–13 million per straw. Based on the weight and concentration, the volume of extender was calculated.

Two *M. bovis* strains were used in spiking: a wild-type isolate from commercial AI straws, strain 198, and reference strain ATCC 27368. The strains were cultured in F broth in closed tubes at 37 °C for 70 ± 2 h. High (10^8 CFU/ml) and low (10^5 CFU/ml) concentration stock solutions were prepared. To verify the *M. bovis* concentration of the stocks, tenfold dilutions were prepared and plated on F plates. The plates were incubated at 37 °C in 5% CO2 for 7 days and colony-forming units were counted.

Semen from the three bulls was pooled and divided into 30 aliquots, which were kept at 32 °C. A commercial animal protein-free extender base containing 7% glycerol was used in all protocols. Six antibiotic protocols were compared: 1) GTLS (500/100/300/600 µg/ml, respectively) fresh antibiotic-supplemented extender; 2) raw semen treated with GTLS fresh antibiotics for 3 minutes and further extended with GTLS (500/100/300/600 µg/ml, respectively) fresh antibiotic-supplemented extender (according to Certified Semen Services (CSS) requirements), later called CSS GTLS; 3) GTLS (250/50/150/300 µg/ml, respectively) antibiotic-supplemented extender (ready-to-use liquid concentrate containing antibiotics), according to the OIE code, Article 4.7.7, later called EU GTLS, 4) ofloxacin 100 µg/ml (Sigma Aldrich 33703) antibiotic-supplemented extender or 5) ofloxacin 400 µg/ml antibiotic-supplemented extender, and 6) extender without antibiotics as a control. The final concentration of the *M. bovis* strains in extended semen was either 10^6 CFU/ml or 10^3 CFU/ml. F broth was used as a negative control in each antibiotic/extender aliquot. All extenders, antibiotics, and F broth were kept at 32 °C before adding to semen. All protocols except number 2 (CSS GTLS) included dilution of the semen 1:1 in extender (with or without antibiotics) and Friis broth containing either 10^8 or 10^5 CFU/ml *M. bovis* ATCC or wild type. In protocol 2 (CSS GTLS), GTLS was first diluted 1:4 in sterile water and 38 µl was added to neat semen (380 µl) and *M. bovis* culture (118 µl) yielding the same antibiotic concentration as if 20 µl GTLS mixture (500/100/300/600) would have been added directly to raw semen. After 3 min incubation at 32 °C, the semen was further diluted 1:1 with extender containing GTLS. All aliquots were then incubated for one hour at 34 °C, after which they were further diluted with extender with or without antibiotics to give a final concentration of 56 million sperm cells/ml. The temperature of the aliquots was then allowed to stabilize to room temperature (approximately one hour), after which an automatic semen straw filling and sealing machine (MPP Quattro, Minitube, Germany) was used. Semen was packed into 0.25-ml straws. After packing, the straws were cooled to 17 °C for one hour and further cooled rapidly to 4 °C. The straws were kept at 4 °C overnight and deep-frozen in an

industrial semen straw freezer (Digitcool 5300, IMV, France) the next morning. Cryopreserved straws were stored in a liquid nitrogen storage tank (-196 °C) until analyzed.

After five weeks of storage in liquid nitrogen, 18 straws from each of the 30 trial lots were randomly retrieved from the nitrogen tank. They were divided into three pools each consisting of six straws. The straws were thawed and the content of the six straws was pooled. From each pool, 0.6 ml of semen was used in three different DNA extraction procedures described in section 4.3, and 0.3 ml of semen was placed into 2.7 ml of F broth. Ten-fold dilutions up to 10^{-5} were prepared in F broth in tightly closed tubes. The broth cultures were incubated at 37 °C for 14 days. From each trial lot, all broth culture dilutions from 10^{-2} to 10^{-4} were tested for *M. bovis* at the latest immediately after the 14-day incubation period.

4.8 Determination of MIC

MICs were determined using custom-made Sensititre plates (Thermo Fisher Scientific, United Kingdom). The antibiotics tested were tylosin (concentration range 0.5–32 µg/ml), lincomycin (0.25–32 µg/ml), spectinomycin (2–128 µg/ml), enrofloxacin (0.03–2 µg/ml), and danofloxacin (0.03–2 µg/ml). Testing was performed according to Ayling et al. [282] and Heuvelink et al. [283]. Briefly, a suspension containing 5% growth indicator alamarBlue (Thermo Fisher Scientific, United Kingdom) in F broth without antibiotics and *M. bovis* 5 x 10^5 CFU/ml was prepared and 200 µl of the suspension was pipetted into each well of the Sensititre plates. The plates were sealed and incubated at 37 °C for 48 ± 1 h and read visually, with a blue color indicating no growth and red indicating growth of the isolate. The MIC was the lowest concentration of antibiotic completely suppressing growth (blue color).

4.9 Statistical analyses

In study I, all statistical analyses were carried out using GraphPad Prism version 7.02 (GraphPad, San Diego, CA, USA). The significance of the differences in the proportions of positive animals at each visit in each infection group was calculated using one-way ANOVA and Tukey's multiple comparison test. $P < 0.05$ was considered significant. The median herd size was calculated, and Pearson's correlation coefficient was calculated to assess the association between herd size and the infection status of the farms, and the association between the BTM anti-MilA and serum anti-MilA antibody concentrations of the cows.

In study III, the agreement among sampling and detection methods was evaluated by calculating the proportion of positive agreement (PPA), the kappa

coefficient, and the corresponding p-value for kappa using Epitools Epidemiological Calculators [319]. The kappa coefficient was interpreted according to McHugh [320]: 0–0.20 no agreement, 0.21–0.39 minimal, 0.40–0.59 weak, 0.60–0.79 moderate, 0.80–0.90 strong, and above 0.90 almost perfect agreement. To determine whether NS and NP sampling differed significantly in the ability to assess a herd visit as positive, McNemar's chi-squared test was conducted. Significance was set at $P < 0.05$.

5 RESULTS

5.1 Characteristics of *M. bovis* infection in the study herds A to S (I)

The study farms A to S were examined for *M. bovis* over the two-year study period using several sampling and study methods. Table 8 presents the size of the herds, the type of index case, the number of *M. bovis* mastitis cases detected in the herds, and the number of milk and post-mortem samples collected by the farmers.

The index case was clinical *M. bovis* mastitis on 17 farms and calf pneumonia on two farms. No cases of mastitis caused by *M. bovis* were detected over the two-year study period on these two farms. The herd size varied from 18 to 268 cows. The median size of the herds was 61 cows and there was a positive, moderate correlation between the herd size and the infection status of the farm (Pearson r = 0.6). In total, 3268 quarter milk samples were tested during the two-year period. Only 51 cows out of approximately 1600 cows on farms A to S had *M. bovis* mastitis. The highest apparent *M. bovis* mastitis prevalence of 7% was observed in herd I, and a prevalence of 4.4% was seen in herds C and N, whereas in eight herds, only one case was detected. Mastitis cases mainly (88%) occurred within the first eight weeks after the index case (I, Table 4). On most farms, cows with *M. bovis* mastitis were isolated and slaughtered or culled as soon as possible after the detection of infection. A total of 22 samples from 10 farms were submitted for post-mortem examination. *M. bovis* was isolated from 12 of these samples, from seven farms. All *M. bovis*-positive post-mortem samples were taken before sampling visit 2.

Table 9 presents the infection status of each of the herds, the number of calves from which NS and NP (only 4 to 6 calves per herd per visit) samples were taken and the percentage of *M. bovis*-positive calves. As most (48/51) *M. bovis* mastitis cases and all *M. bovis*-positive post-mortem samples were detected between the time from the index case and sampling visit 2, the grouping of herds into different infection status groups was based on the detection of *M. bovis* in NS and/or NP samples from calves. On infection status S0 farms (n = 2, herds K and M), *M. bovis* was only detected in the index case and was not isolated at any other time throughout the study period. On four farms (infection status S1, herds J, O, P, and Q), *M. bovis* was only detected in the first half of the first study year, and on two farms (infection status S2, herds B and F), it was detected throughout the first year of the follow-up period. In 11 herds (infection status S3, herds A, E, I, and N; infection status S4, herds C, D, and S; and infection status Sx, herds G, H, L, and R), *M. bovis* was detected after the first year, and the majority of these farms had large herds in loose housing barns. The results of NS rtPCR and NP culture from herds A to S are presented in study III, in which these herds are coded as herds 1–19.

Table 8. Description of herds and number of milk and post-mortem samples collected by farmers.

					Sample types, method, no. of samples tested for *M. bovis*			
					QMS[2]		Bulk tank milk	Post-mortem
Herd ID	No. of cows	Barn type	Index case[1]	Total no. of mastitis cases	PCR	PCR	ELISA	Culture
A	47	Tie-stall	M	1	64	16	20	
B	61	Loose housing	M	2	252	16	15	2
C	183	Loose housing	M	8	154	4	6	2
D	268	Loose housing	M	6	500	2	6	
E	25	Loose housing	M	1	27	16	12	
F	50	Tie-stall	M	1	28	18	21	1
G	157	Loose housing	M	1	487	19	19	
H	60	Loose housing	M	4	194	17	20	3
I	100	Loose housing	M	7	96	17	16	
J	61	Loose housing	M	3	595	22	20	
K	29	Tie-stall	M	1	51	12	21	1
L	41	Tie-stall	M	1	51	6	6	
M	66	Loose housing	M	1	112	18	19	1
N	158	Loose housing	M	7	309	15	11	4
O	48	Loose housing	CP	0	94	13	9	1
P	18	Tie-stall	M	2	12	11	18	
Q	28	Tie-stall	M	1	30	18	19	
R	66	Loose housing	CP	0	36	12	19	1
S	127	Loose housing	M	4	176	11	8	6

[1]M, mastitis, CP, calf pneumonia; [2]Farmers were advised to monitor cattle for mastitis and other clinical signs thoroughly and to submit samples for testing for mastitis pathogens in all cases of subclinical and clinical mastitis by quarter milk sampling. Some farmers also submitted samples from clinically normal animals.

Table 9. Number of studied calves per herd per sampling visit 1 to 4, and percentage of *M. bovis*-positive calves. A calf was *M. bovis*-positive if the rtPCR from NS and/or culture from NP was positive.

Herd	Infection status	No. of studied calves 1	% positive calves 1	No. of studied calves 2	% positive calves 2	No. of studied calves 3	% positive calves 3	No. of studied calves 4	% positive calves 4
K	S0	15	0	9	0	10	0	10	0
M	S0	9	0	16	0	18	0	13	0
J	S1	20	75	18	0	27	0	20	0
O	S1	16	25	21	0	19	0	15	0
P	S1	9	11	10	0	9	0	8	0
Q	S1	20	10	15	0	11	0	12	0
B	S2	19	58	26	12	23	0	19	0
F	S2	13	31	18	17	14	0	13	0
A	S3	19	11	15	20	23	4	19	0
E	S3	15	60	10	10	14	43	14	0
I	S3	20	15	18	50	20	5	20	0
N	S3	23	48	26	61	20	55	21	0
C	S4	21	52	20	60	19	21	20	5
D	S4	54	46	20	50	20	10	23	35
S	S4	14	64	20	35	20	25	15	7
G	Sx	20	50	20	0	19	16	20	0
H	Sx	16	31	19	53	14	0	19	5
L	Sx	21	33	20	0	20	45	16	44
R	Sx	20	25	21	0	18	0	21	62

5.2 Serum antibodies against *M. bovis* in cows and young stock (I)

A total of 3017 serum samples from herds A to S were tested using two different ELISAs, using a cut-off value 135 AU for the MilA ELISA and an ODC of 37% for the K 260 ELISA. MilA ELISA detected (I, Table 5).

The *M. bovis*-specific antibody profiles for each infection status group using both ELISAs were analyzed (Figures 2 and 3). The proportions of MilA ELISA-positive young stock (under 2 years of age) followed the proportions of *M. bovis*-positive calves detected by PCR of NS and culture of NP samples. In contrast, no such patterns were observed with the Bio-X ELISA. There was a significant decrease in the proportion of young stock that were positive in the MilA ELISA after the first sampling visit ($P < 0.05$) on S0 and S1 farms (Figures 2 a, b). Similarly, there was a significant decrease in the proportion of young stock that were positive in the MilA ELISA after the second visit on S2 farms ($P < 0.05$, Figure 2 c). In contrast, on S3, S4, and Sx farms, the proportion of young stock that were positive

in the MilA ELISA remained as high as 80% at most time points, without any significant decrease in the proportion that were positive over the duration of the study (Figures 2 d, e, f). However, 80% to 100% of cows were MilA ELISA-positive throughout the duration of the study, regardless of the infection status of the farm. There were a few exceptions at a few time points, but no significant differences between the time points. On S0 farms, there was a significant decrease in the proportion of positive cows after the first visit, but the proportion had increased again by the third visit. On S4 and Sx farms, all the cows tested were positive in the MilA ELISA at the start of the project, and approximately 80% were positive at each time point thereafter (Figures 2 e, f). No patterns were detectable in the Bio-X ELISA results from the different infection status groups (Figure 3). However, there was a significant decrease in the proportions of young stock and cows on S4 farms that were positive in the Bio-X ELISA after the first sampling (Figure 3 e).

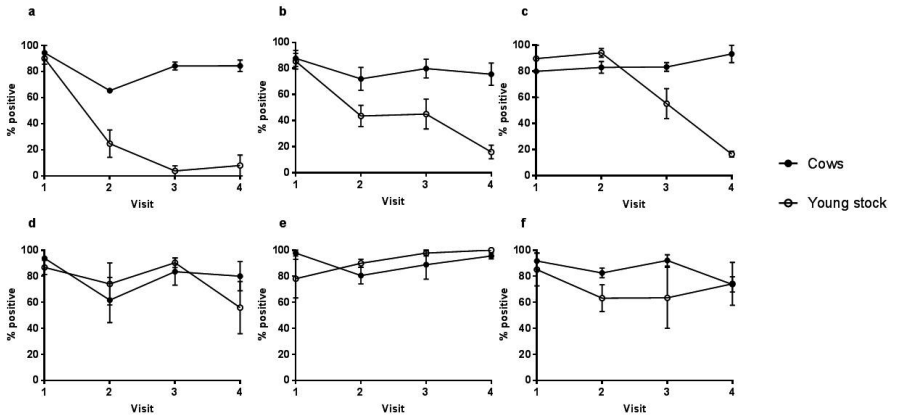

Figure 2. Proportion (percentage) of MilA-seropositive samples in cows and young stock (<2 years of age) in different infection status groups. a = S0, b = S1, c = S2, d = S3, e = S4, f = Sx.

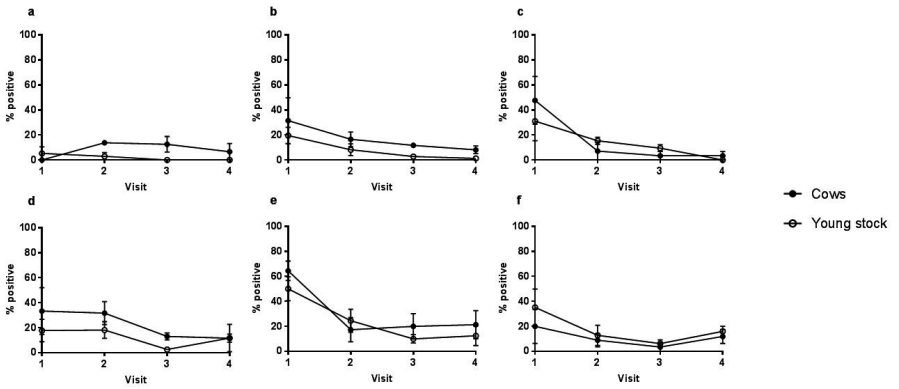

Figure 3. Proportion (percentage) of BioX ELISA-seropositive samples in cows and young stock (<2 years of age) in different infection status groups. a = S0, b = S1, c = S2, d = S3, e = S4, f = Sx.BTM samples: PCR and serology

The number of BTM samples collected by farmers for BTM PCR varied between farms from 2 to 22 and for serological testing from 6 to 21 during the two-year follow-up period. Altogether, 263 BTM samples yielded seven *M. bovis*-positive samples from five farms. All these positive samples, except one, were collected within four weeks of the index mastitis case in the herd. On farm E, a single BTM sample was positive 5 months after the index case. After this positive finding, quarter milk samples from cows on farm E with an elevated somatic cell count were tested for *M. bovis,* and all were negative. Two cows with subclinical mastitis were dried off in the meantime without sampling.

Anti-MilA antibodies over 135 AU were detected in only one BTM sample out of 285 samples tested, and all other samples were negative. In contrast, when BTM samples were tested using K260 ELISA, altogether 20 samples were positive (ODC% ≥ 37). Eighteen of these positive samples had been taken 20 to 96 days after the index mastitis case (Table 10). On farm E, with PCR positive BTM at the beginning of August 2015, the BTM sample for serology had been negative at the end of July, but the next sample after that, taken in October, was positive in K260 ELISA. There was a positive correlation between the anti-*M. bovis* antibody concentrations in the BTM samples (n = 68) and those in sera of the cows (Pearson r = 0.45), as measured using the MilA ELISA (I, Figure 3).

Table 10. Bio K260 and MilA BTM results.

Farm	Cows	Index case	BTM 1	K260	MilA	PCR	BTM 2	K260	MilA	PCR
A	47	29.4.2014	10.11.2014	0	10	neg	17.12.2014	0	12	neg
B	61	21.4.2015	19.5.2015	45	76	neg	25.11.2015	16	13	neg
C	183	14.1.2016	16.2.2016	94	120	neg	24.11.2016	9	16	neg
D	268	12.8.2013	8.9.2015	19	13	neg	24.11.2016	23	23	neg
E[#]	25	10.3.2015	30.3.2015	47	98	neg	7.5.2015	28	4	neg
F	50	12.5.2015	27.5.2015	66	43	neg	24.6.2015	55	92	neg
G	157	15.1.2016	24.2.2016	22	16	neg	31.3.2016	29	24	neg
H	60	5.11.2015	14.12.2015	47	49	neg	11.1.2016	21	21	neg
I[&]	100	23.12.2015	3.2.2016	50	43	pos	7.3.2016	56	51	neg
J	61	11.12.2015	11.1.2016	116	110	pos	1.2.2016	69	74	neg
K	29	12.7.2014	11.11.2014	0	27	ND	15.12.2014	0	13	neg
L	41	24.5.2014	27.11.2014	0	32	neg	15.1.2015	23	12	neg
M	66	29.8.2014	12.11.2014	0	29	neg	1.1.2015	20	19	neg
N[$]	158	18.3.2015	15.4.2015	50	107	neg	16.11.2015	29	19	neg
O	48	31.3.2015	28.4.2015	14	26	neg	1.6.2015	11	33	neg
P	18	29.10.2015	25.11.2015	54	301	pos	7.12.2015	27	64	neg
Q*	28	12.8.2015	9.9.2015	48	36	neg	7.10.2015	35	43	neg
R	66	1.9.2015	20.10.2015	21	18	neg	9.11.2015	22	23	neg
S[§]	127	25.1.2016	9.2.2016	96	64	pos	29.3.2016	39	33	neg

[#]5.8.2015 BTM PCR-positive, 16.10.2015 BTM PCR-negative, K260 38; [&]4.4.2016 K260 41; [$]10.8.2016 K260 38; *3.11.2015 K260 38; [§]15.8.2017 K260 47

5.3 *M. bovis*-contaminated AI semen as a source of infection (II)

Farms X and Y were both loose barn dairy farms with good or excellent biosecurity. Farm X had been a closed herd since 2011 and Y since 2003, they did not use contract heifer rearing, and they had only used domestic embryos. Both farms regularly monitored QMS for pathogens, including *M. bovis*. The distance to the closest cattle farm was 1.2 km for farm X and 1.6 km for farm Y. Altogether, seven cows developed *M. bovis* mastitis in these two herds and 10 different bulls had been used to inseminate these cows. Semen lots tested from bulls B to J were negative (II, Table 4.). Semen from lots 3 and 4 from bull A had been used on both farms approximately 32 days earlier to inseminate the cows that were the first ones that developed *M. bovis* mastitis (II, Figures 1 and 2). Further examination of 22 different semen lots from bull A revealed live *M. bovis* in lots 3 and 4, and lots 6 and 12 were positive in PCR but negative in culture (II, Table 5). Culture and PCR results revealed that *M. bovis* was not evenly distributed in the straws, even within same lot, and the bull seemed to shed the bacteria for only a short time and intermittently (II, Table 5). The *ad-hoc* cgMLST schema covered 58.2% of the PG45 reference genome. From the 11 isolates in this study, 589 cgMLST allele-called targets were extracted and compared with each other. Except for the Estonian isolate strain 537, all Finnish isolates clustered together within an allele difference of 2–24. The mastitis strains from farms X and Y and the bull semen strain had allele differences of 4 and 8, respectively, and clustered together (II, Figure 3).

5.4 *M. bovis* in NS and NP samples from calves in recently infected dairy herds (III)

The number of cows in the herds of study III varied from 18 to 315, the mean being 91 cows, and 9/30 herds had 100 or more cows (III, Table 1). The total number of NS taken from 3- to 348-day-old calves in 30 herds recently infected with *M. bovis* was 1037. The overall apparent *M. bovis* prevalence in NS samples was 29.5%. The highest prevalence of 43% was detected in calves aged 31 to 60 days. Thereafter, shedding decreased and was 13.7% in 150- to 180-day-old calves (III, Table 3). Large variation from zero to 75% was seen between the herds in the apparent prevalence of nasal shedding. Both NS and NP samples were taken from 284 calves. *M. bovis* was detected in 93/284 (32.7%) and in 133/284 (46.8%) of NS and NP samples, respectively. The proportion of positive agreement of NS

compared to NP samples in these calves was 0.68 and the kappa coefficient was 0.48 (weak) (III, Table 2).

In calves suffering from acute BRD, *M. bovis* was detected in 29/62 (47%), 24/62 (38.7%), 15/62 (24.2%), and 14/62 (22.6%) of BAL, NP, NS (culture), and NS (real-time PCR) samples, respectively. The proportion of positive agreement of NP compared to BAL was 0.91 and the kappa coefficient was 0.84 (strong), whereas the proportion of positive agreement of NS (real-time PCR) compared to BAL (culture) was 0.65 and the kappa coefficient was 0.50 (weak) (III, Table 1). Nasal swabs analyzed by culture only yielded one more positive sample compared to PCR.

5.5 Effectiveness of NS and NP samples in indicating the *M. bovis* infection status of dairy herds (III)

Altogether, 89 sampling visits were carried out in herds 1 to 30. In herd 4, during sampling visit 1, only NS samples were taken from calves. These were not included in the analysis. All samples from two herds were already negative at the first visit (herds 11 and 13). Otherwise, there were 54 herd visits out 88 visits with a positive infection status, meaning that at least one *M. bovis*-positive NS or NP was found in the herd. Out of the 54 herd visits with a positive infection status, 51/54 (94.4%) would have been classified as infection status positive if only NS had been analyzed, and 43/54 (79.6%) as infection status positive if only NP samples had been analyzed (III, Tables 4 and 5). This difference was not statistically significant ($p = 0.061$).

5.6 Efficacy of antibiotics in semen extender against *M. bovis* (IV)

Raw pooled semen showed no growth in mycoplasma culture. *M. bovis* or F broth did not show any detrimental effect on quality parameters of the semen (IV, Table 1). After storage of the AI straws for five weeks in liquid nitrogen, at high spiking concentrations (10^6 CFU/mL), viable *M. bovis* bacteria were detected in processed semen, regardless of the processing protocol. When low *M. bovis* concentrations were inoculated, differences among processing protocols were seen (IV, Table 2). At a low spiking concentration, the ATCC strain was more resistant than the wild-type strain to different antibiotics. The only protocol inhibiting the growth of the ATCC strain was the high GTLS 500/100/300/600 µg/mL (final concentration in extended semen) supplement added in the semen lab to the extender. All protocols, except EU GTLS 250/50/150/300 µg/mL (final concentration in extended semen)

and extender without antibiotics, inhibited the growth of the wild type at a low spiking concentration. Antimicrobials present in extended semen affect the mycoplasma culture, and several dilutions were thus made. In samples with a high concentration of antimicrobials, viable *M. bovis* could only be detected in the highest culture dilution (IV, Table 2). The strains used in spiking were sensitive for the antibiotics tested according to CLSI SIR values for *Pasteurellaceae* in cattle (table 11).

Table 11. MIC values (µg/mL) of ATCC 27368 and wild-type strains (dilution range of antibiotic tested).

Antibiotic	Dilution range tested (µg/mL)	ATCC 27368	Wild type
Tylosin	0.5–32	≤0.5	16
Lincomycin	0.25–32	2	1
Spectinomycin	2–128	4	≤2
Enrofloxacin	0.03–2	0.25	0.25
Danofloxacin	0.03–2	0.25	0.25

5.7 Methods to isolate *M. bovis* DNA from extended semen (IV)

We compared three different DNA extraction methods for spiked semen samples. At a high spiking concentration (10^6 CFU/mL), all pools were positive in PCR, regardless of the DNA extraction method. Ct values varied between 24.7 and 28.5, and no significant differences in Ct values among extraction methods were seen. At a low spiking concentration, the method using InstaGene™ (method three) was the most effective. Using this method, we detected *M. bovis* in 94% (17/18) of pools spiked with 10^3 CFU/mL of the ATCC strain, and in 72% (13/18) spiked with 10^3 CFU/mL of the wild-type strain. With method 1, 67% (12/18) and with method 2, 56% (10/18) of pools spiked with the ATCC strain were positive in PCR. For the wild-type strain, the respective figures were 61% (11/18) for method 1 and 33% (6/18) for method 2 (IV, Table 3) The Ct values varied between 34.3 and 36.7, and no significant differences in Ct values among extraction methods were seen.

6 DISCUSSION

6.1 Characteristics of *M. bovis* infections on Finnish dairy farms (I)

In most of our study herds, the index case was clinical mastitis. No actual outbreaks of *M. bovis*-associated diseases were observed. Ten herds experienced only one to two mastitis cases during the two-year follow-up period. The highest apparent within-herd prevalence of *M. bovis* mastitis was from 4.4% to 7% on three farms. Information in the literature on the within-herd prevalence of *M. bovis* clinical mastitis is scarce. In Japan, Murai et al. [321] detected a 2.8% within-herd prevalence of *Mycoplasma* IMI and Timonen et al. [164] estimated it to be from 3.7% to 11% in four Estonian herds. Lysnyansky [322] reported that 90% of *M. bovis* mastitis herds in Israel had less than ten cases within a herd, and Pinho et al. [115] found the within-herd mycoplasma prevalence to vary from 2.7% to 4.5%. Others have reported devastating outbreaks in which up to 50% to 100% of cows have been culled due to mastitis, arthritis, and pneumonia caused by *M. bovis* [83,323]. Dairy herds in Finland have a long tradition of bacteriological testing of QMS for clinical and subclinical mastitis. Since spring 2012, this multiplex PCR has included primers and a probe specific for *M. bovis*. Rigorous testing of QMS from mastitis cows has probably aided in the early detection of *M. bovis* in our study herds. All cows with *M. bovis* mastitis were isolated and culled after a positive result from PCR. The early detection and culling of infected cows could have prevented actual outbreaks in the study herds. *M. bovis* is considered a contagious mastitis pathogen that spreads from udder to udder during milking. Thus, several studies have emphasized the removal of infected cows from the lactating herd as an important control element for mycoplasma mastitis [42,61,324]. On the contrary, Punyapornwithaya et al. [325] found that culling of mycoplasma mastitis cows did not affect the time to clearance of a *M. bovis* mastitis episode, as 78% of the studied dairy herds cleared the episode within one month, and only half of these herds opted for culling of infected cows. Nevertheless, *M. bovis* mastitis is generally considered to be incurable with antibiotics, can lead to permanently reduced milk production, and once infected, cows are advised to be kept for the rest of their life in their own separate milking group. Thus, culling is often the most feasible choice [54,61].

In two herds, the index cases were pneumonia in calves, but no *M. bovis* mastitis was detected during the two-year follow-up period. Prolonged colonization that lasted over one year in young stock was seen in 11 out 19 herds. The possible role of colonized calves as the nidus for *M. bovis* mastitis has been speculated [61,324].

Amran et al. [173] demonstrated that in a dairy herd that had bought imported calves three months earlier, severe pneumonia followed by mastitis occurred in cows, and genotyping revealed that isolates from the diseased cows were identical to *M. bovis* strains isolated from the imported calves during quarantine. They concluded that the calves were the source of respiratory infection of cows and that spread from the lungs to the udder occurred. In a Danish risk factor study, contact of young calves with older age groups was also a significant risk factor for *M. bovis*-associated disease in cows [86]. This does not appear to apply to Finnish dairy herds, because in spite of widespread and prolonged colonization of calves on many farms, almost all detected mastitis cases occurred within eight weeks from the index case. Finnish cow barns often house cows and calves in the same barn, sharing the airspace. This was also the case in 14 of our study herds.

Serum antibody responses were very different when results from the commercial Bio-X K 260 and the in-house MilA ELISA were compared. Of all tested serum samples, 15.5% and 76.1% were positive using K 260 and MilA, respectively. As already stated in the background section, from the beginning of studies on *M. bovis* infections in Finland, we had suspicions that the sensitivity of neither the Bio-X K 302 nor the K 260 ELISA was high. However, when this project was started in 2014, Bio-X ELISA tests were the only ones commercially available. We chose to use the K 260 test because each sample is also tested in a negative control well, which should increase specificity. In a sensitive and specific ELISA test, the antigen used needs to be highly conserved among isolates from different countries, needs to be expressed during infection, and has to be immunogenic as well as specific to the studied pathogen. The manufacturer of the K 260 test holds the identity of the antigen as proprietary knowledge. Thus, it is impossible to state whether the seemingly low sensitivity of K 260 is due to an antigen not expressed stably by Finnish *M. bovis* strains or if the antigen has low immunogenicity. Studies published after the initiation of our project have demonstrated that K 260 seems to be very specific (100%), but its sensitivity is low (13% to 28%) [237,239]. Both studies have used sera from experimentally infected young calves, so field studies sampling cattle with different *M. bovis*-associated diseases, as well as animals from herds known to be free of *M. bovis*, are needed to properly understand the usefulness of the K 260 ELISA.

MilA has been shown to be a membrane protein with lipase activity, and antibodies against MilA have been constantly detected in the sera of experimentally infected calves [238,239]. Subsequently, Adamu et al. [28] observed that MilA binds both triglycerides and fatty acids and has lipase activity, and they found indications that MilA acts as an autotransporter. It was also demonstrated to bind to heparin, a component of the extracellular matrix, thus indicating it has a role as an adhesin. A transposon mutagenesis study did not find any insertions in the *milA* gene, indicating that it might be an essential gene in *M. bovis* [326]. These findings suggest that MilA is probably highly conserved in different *M. bovis*

strains, and it has been shown to be immunogenic, making it a good candidate antigen for serological testing.

Our MilA antibody results indicate that once *M. bovis* enters a herd, it rapidly spreads to all age groups. It appears that the MilA ELISA is so sensitive that it even measures exposure to *M. bovis*, as 80% to 100% of blood samples taken during sampling visit one were positive, although no clinical disease was observed in such high proportions of animals. It has to be kept in mind that anti-MilA results from different ELISA plates might display some variability, as this is an in-house ELISA in which plates are coated in the laboratory and each plate has its own standard curve. In young stock, the proportion of MilA-positive animals followed the pattern seen in the proportion of *M. bovis* URT-colonized calves. Interestingly, the proportion of MilA-positive cows remained high in all herds for at least one and half years, even in herds that appeared to have resolved the infection. Adamu et al. [28] stated that MilA homologues can be found in several *Mycoplasma* species, including *M. bovigenitalium*, *M. canadense*, and *M. canis*, which have all been found to occasionally cause mastitis and other infections in cattle. Field studies similar to those presented for K 260 are needed to assess the specificity of the MilA ELISA.

The detection of *M. bovis* in BTM using rtPCR has been shown to be a sensitive method with a limit of detection 10^2 CFU/ml [189,215,219]. Theoretically, if a cow excretes 10^8 CFU/ml of bacteria, rtPCR should detect *M. bovis* in BTM if one out of 300 cows is excreting and milked into the tank. In our study, *M. bovis* was only detected in a BTM sample in five herds. In four herds, these positive samples had been taken 15 to 41 days after the detection of the index case. All these herds had 2 to 7 *M. bovis* mastitis cases. Routine BTM rtPCR screening for *M. bovis* does not appear to be cost-efficient in Finland, because individual cow QMS testing for mastitis pathogens is so widely used. This leads to the early detection of cows with *M. bovis* mastitis and their removal from the herd.

In contrast to rtPCR detection, antibodies against *M. bovis* were detected in 11 out 17 herds with *M. bovis* mastitis using the Bio-X K 260 ELISA. No BTM antibodies were detected in two herds with calf pneumonia as the main manifestation. The K 260 ELISA detected antibodies in samples taken 20 to 96 days after the index case. Five out of the six mastitis herds that were negative in BTM serology sent the first sample for serology at the earliest two months after the detection of the index case. Unfortunately, the farmers did not always follow the instruction to send a BTM sample for serology once a month. This hindered us in properly following the antibody dynamics in BTM. Our results reflect the K302 BTM ELISA responses seen in Denmark, where Petersen et al. [240] concluded that the antibody response in BTM is very dynamic and short-lived. In Australia, Parker et al. [241] found, using the K302 ELISA, that antibodies could be detected in BTM from one to a maximum of eight months after exposure. Thus, BTM serology using Bio-X ELISA tests appears to be an unreliable method for use

in biosecurity unless sampling is repeated continuously at least once a month. Petersen et al. [240] sampled BTM from herds with various *M. bovis*-associated diseases and found that not all clinical symptoms (e.g., arthritis) in cows are reflected in the BTM, and neither is the disease situation in young stock. Thus, BTM serology can only be used to detect (previous) *M. bovis* mastitis or subclinical mastitis in a herd.

MilA and K 260 BTM antibody results were very different, as only one sample was positive in the MilA ELISA and all other samples were clearly below the cut-off. Ours is the first study describing the use of the MilA ELISA to detect *M. bovis* antibodies in BTM. It is possible that the ELISA procedure was not optimal, thus affecting the results. However, a positive, moderate correlation between serum and BTM MilA antibody units warrants further development and studies on the MilA response in BTM.

6.2 New transmission route: contaminated AI semen (II)

Our epidemiological investigations did not reveal any other source than *M. bovis*-contaminated semen for *M. bovis* infection in two closed dairy herds. Results from cgMLST analysis supported this. Internationally traded semen is not a recognized pathway for *M. bovis* transmission. *M. bovis* has previously been cultured from commercial semen in Israel [173,269], but wgSNV analysis of semen and mastitis isolates did not cluster them together [269]. *M. bovis* can colonize the female reproductive tract following insemination with experimentally infected semen [95]. Next to nothing is known about the number of bacteria in naturally contaminated semen or the dose of bacteria needed to initiate an infection in a cow or heifer through insemination. Thus, significant uncertainty relates to the likelihood of transmission, even if semen is contaminated with *M. bovis*. However, when Haapala et al. [304] examined the risk factors for the introduction of *M. bovis* infection into naïve herds in Finland, they found in univariable risk analysis that the use of contaminated semen lots in AI was the most significant risk factor ($P = 0.004$).

Nowadays, genomic selection methods allow the identification of potential AI bulls within weeks of birth. A tendency in AI centers is to collect semen from young bulls as early as possible after they have reached sexual maturity. It is estimated that nowadays, over 50% of bulls whose semen is marketed are younger than 15 months of age [327]. This can also pose a risk regarding infectious diseases such as *M. bovis* infection, because young animals are often more prone to infectious diseases, and this can lead to the secretion of bacteria into semen. The contaminated semen in this case had been collected in Denmark from a 9-month-old bull. Recently, García-Gálan et al. [328] reported that mycoplasma horizontal chromosomal transfer can occur in *M. bovis* under field conditions,

probably during coinfection of cattle with multiple *M. bovis* strains. Although this is expected to happen at low a frequency, this phenomenon contributes to the genome-wide variety in *M. bovis*. Mycoplasma horizontal chromosomal transfer affects both housekeeping genes and the accessory genome and can alter gene expression [329]. This can lead to new types of strains with higher infectivity, or to strains towards which cattle show a weak or non-existent immune response. In Denmark, *M. bovis* was isolated for the first time in 1981 [330]. Subsequently, little attention was paid to *M. bovis* in Denmark, although it was detected in calves with respiratory disease and in some mastitis outbreaks. At the end of 2011, sudden and severe *M. bovis* outbreaks occurred on dairy farms. A molecular epidemiological study revealed that these outbreaks were caused by a new clone [263]. Further detailed genomic studies are needed to examine the possible role of mycoplasma horizontal chromosome transfer in the development of this new clone. However, it is possible that this new clone efficiently colonized or even caused disease in young animals and led to *M. bovis* secretion into semen.

Our study demonstrated that the bull intermittently shed *M. bovis* into semen for only a short time, altogether for seven weeks. The bull was tested for carrier status 17 months after the beginning of semen collection, but no *M. bovis* was found in semen or mucosal swabs. It appeared that *M. bovis* was not evenly distributed in extended semen, because it was not found in all straws studied from the same lot. High Ct values also indicated that there were low levels of bacteria in positive lots, which could mean that in some straws the amount of *M. bovis* was below the level of detection. *M. bovis* growth was only observed in the 10^{-3} culture dilution. In lower dilutions, the antibiotics added to the extender inhibited the growth of *M. bovis*. These results support the presumption that semen extender antibiotics merely have a mycoplamastatic effect and do not entirely eliminate *M. bovis* in semen. The results indicate that the reliable detection of *M. bovis* in extended semen can be difficult and several straws from a lot need to be examined.

Interestingly, in both herds, the cows inseminated with contaminated semen developed clinical *M. bovis* mastitis 32 days after insemination. We can only speculate about how infection spread from the genital tract to the udder. It could have been through vaginal discharge passing via the teat canal to the udder or could have been a hematogenous spread from the genital tract to the udder. Either way, the incubation period was approximately one month.

6.3 Suitability of NS and NP sampling of calves in a control program (III)

When the Finnish *M. bovis* control program was designed, regular NS sampling of calves younger than six months of age was included as one part of the program.

There have been only a few reports on the prevalence of nasal shedding of *M. bovis* in dairy calves. Bennet and Jasper [9] observed that 34% of calves in herds with *M. bovis* mastitis shed the bacteria into nasal secretions compared with 6% of calves in non-problem herds. A significantly lower nasal prevalence of 2.4% in weaned calves in dairy herds with recent *M. bovis* mastitis was seen in Australia [191]. Knowledge of *M. bovis* nasal shedding in dairy calves in herds with recently detected *M. bovis*-associated disease is scarce, but this is important for assessing the usefulness of nasal swabbing of young calves as part of a control program. In our study herds, the overall *M. bovis* prevalence in NS was almost 30%. The highest nasal shedding rate was seen in 31- to 60-day-old calves, whereafter the shedding rate steadily decreased. Only a small number of NS samples were taken from calves older that five months of age, so some caution is needed regarding the prevalence in older calves. Although we used rtPCR and Bennet and Jasper [9] used mycoplasma culture, the results are very similar.

Maunsell et al. [92] demonstrated that after feeding calves *M. bovis*-contaminated milk, both palatine and pharyngeal tonsils were the main site of *M. bovis* colonization, but only a few calves were found to shed *M. bovis* into nasal secretions. Buckle et al. [331] examined swabs taken post-mortem from the palatine tonsils of healthy calves that originated from a *M. bovis*-seropositive herd. Real-time PCR detected *M. bovis* in 93% of the tonsillar swabs, whereas only 12% of tracheal swabs were positive. These findings suggest that tonsils, rather the nasal epithelium, are the main upper respiratory tract colonization sites, and tonsillar swabs are therefore the most sensitive sampling method to detect *M. bovis* carriers among calves. Indeed, when we compared NS and NP swabs taken simultaneously from 284 calves, 47% of NP swabs were positive compared to 33% of NS swabs. NP swabs sample the respiratory and associated lymphoid epithelium of the nasopharynx, and in live calves are therefore the most comparable technique to post-mortem pharyngeal tonsil swabbing. However, guarded NP swabs are expensive compared with simple bacteriological swabs, and an assistant is needed while taking the sample, making this sampling method less attractive in a control program.

Our study included 54 herd visits that deemed the herd as having a positive infection status, that is, at least one of the analyzed NS and/or NP samples was *M. bovis* positive. Depending on the herd size, 6 to 28 NS samples were taken per herd visit, but the number of NP samples taken was five. Real-time PCR from NS correctly classified 94.4% of infection status positive herd visits as positive, whereas enrichment culture of NP samples followed by rtPCR classified only 79.6% of visits correctly. The reason for this difference is related to the number of samples taken. By taking several NS samples from young calves, the sensitivity of the sampling method increases. The average dairy herd size in Finland is 50 cows [8], and most herds have year-round calving. Bull calves are usually sold to calf-rearing farms at the age of two to four weeks. Considering that *M. bovis*

shedding into nasal secretions can be intermittent or that *M. bovis* can even reside in tonsils without apparent nasal shedding, NS sampling needs to be considered as an imperfect test. As Humphry et al. [332] pointed out, applying an imperfect test in a small herd where the prevalence of latent carrier animals is thought to be low is problematic, as even sampling of the whole herd would not give high confidence in freedom from disease. Despite extensive research, it has not been possible to identify an accurate testing method that can reliably detect *M. bovis* latent carriers and could be used in herd certification or a control program. Antibody testing has demonstrated that antibodies persist in cows for a long time (study I, [333]). Hence, testing of antibodies is not suitable to detect an active infection. Previous studies have demonstrated that after an initial mastitis outbreak, colonization and shedding are not consistently associated with a particular anatomical site and shedding rapidly decreases in cows ([188,190]. *M. bovis* is more prevalent in the URT of calves, and sampling should be targeted at these animals. We conclude that NS sampling from calves under six months of age with analysis by rtPCR is a cost-efficient method to be used as a part of a *M. bovis* control program in dairy herds. In the Finnish *M. bovis* control program, the herd health veterinarian visits each herd biannually and samples the calves. This allows sampling to be targeted at calves younger than six months of age in which the prevalence of *M. bovis* is highest.

6.4 Detection of *M. bovis* from calves with acute BRD (III)

Several sampling procedures, namely NS, NP, BAL (both endoscopic and nonendoscopic), and transtracheal wash (TTW), are used to collect samples for diagnostic tests to identify pathogens involved in BRD ([201,203,204]. A TTW sample is considered to be the golden standard technique. It is thought to best represent the bacterial population in the lower airways but is a time-consuming and invasive method requiring the use of sedation and a local anesthetic. Nonendoscopic BAL samples individual random lung lobes and the sampled surface is large (over 10 cm^2), whereas NP samples a small area (approximately 0.5 cm^2) of the respiratory and associated lymphoid epithelium in the nasopharynx [204]. Both nonendoscopic BAL and NP sampling lead to the contamination of samples from the nasal passages if non-guarded catheters or swabs are used. Guarded BAL catheters are not commercially available in Finland, besides which, BAL sampling can cause respiratory distress in sampled calves due to insufficient aspiration of instilled fluid.

In our study, nonendoscopic BAL using a guarded catheter was the most sensitive method to detect *M. bovis* in pneumonic calves. However, NP had close agreement with BAL sampling in detecting *M. bovis*. Pardon and Buczinski [204] have calculated that taking four to five samples and analyzing them with

a diagnostic test that has 70% Se and 100% Sp leads to almost a zero risk of not finding the pathogen causing the respiratory disease outbreak. In conclusion, guarded NP sampling of four to five calves is a sensitive and practical method to detect *M. bovis* in pneumonic calves.

Doyle et al. [201] demonstrated that the agreement between NS, NP, and BAL compared to TTW was good for *M. bovis*, but more *M. bovis* was isolated from BAL than TTW. In contrast to this study, we found that NS sampling is not a suitable method to detect *M. bovis* in calves with acute respiratory disease, because compared to BAL, the kappa coefficient was weak. Thomas et al. [200] compared NS with BAL to detect *M. bovis* in pneumonic calves under one year of age and found that NS had a sensitivity of only 21%, meaning that NS was not predictive of *M. bovis* in the LRT. Our results agree with this, suggesting that NS cannot be recommended for use as a sampling method to detect *M. bovis* in pneumonic calves.

6.5 Efficacy of antibiotic–extender combinations against *M. bovis* in bovine semen production (IV)

Studies on the effect of antibiotics in bovine semen extenders against *M. bovis* date back to the 1980s and 1990s, when extenders contained proteins from animal sources. We aimed to test the effect of two different GTLS and ofloxacin concentrations using a modern plant protein-containing extender and an industrial-scale semen straw production system. Besides the *M. bovis* PG45 reference strain, we were able to examine the effect of antibiotics on a wild-type strain that we had isolated from commercial semen. Our aim was to determine whether it is possible to achieve a 100% bactericidal effect using GTLS or ofloxacin in semen extender. According to our results, none of the studied antibiotics affected the viability of *M. bovis* at a spiking concentration of 10^6 CFU/mL. At a low spiking concentration of 10^3 CFU/mL, we obtained discrepant results. Growth of the wild-type strain isolated from semen was inhibited by all antibiotics except the low concentration of GTLS, whereas the high GTLS concentration was the only antibiotic treatment that inhibited the growth of the PG45 reference strain.

The most widely used antibiotic combination in bovine semen production is GTLS, originally developed by Shin et al. [311]. They tested different concentrations of antibiotics and concluded that the most effective concentrations against mycoplasmas and bacteria were 500, 100, 300, and 600 µg/mL of GTLS, respectively. This antibiotic combination was first added to raw semen and further to extender. They used a spiking concentration of 10^5 to 10^6 CFU/mL of *M. bovis* and, depending on the extender used, reported a bactericidal effect of 60–80%. The authors' opinion was that although a 100% bactericidal effect had not been

achieved, the reduction in the number of *M. bovis* was so significant that it made the semen safe to use. The protocol of Shin et al. was adopted by Certified Semen Services (CSS) in the US, and the use of this protocol is a requirement for AI semen imported into the US. Later studies by Visser et al. [312,334] demonstrated that although GTLS had an obvious bacteriostatic effect, no significant bactericidal effect was observed. The authors concluded that GTLS in semen was not capable of completely eliminating *M. bovis* in frozen bovine semen. For reasons unknown to the author, the GTLS concentration stated in the regulation EU 2020/686 and the OIE Terrestrial Code is half of that reported to be most effective by Shin et al. [311].

There is substantial evidence for increasing antibiotic resistance among *M. bovis* strains, including increased macrolide and linco/spectinomycin resistance. This may impact on the effect of GTLS on *M. bovis* in semen. MIC studies have indicated that most contemporary *M. bovis* isolates are susceptible to fluoroquinolones. Gloria et al. [335] observed that ofloxacin, a fluoroquinolone antibiotic, did not have any negligible effect on spermatozoa up to a tested concentration of 400 µg/mL. Ofloxacin at a concentration of 100 µg/mL in semen extender resulted in a significant reduction in the bacterial concentrations, with 90% of samples showing complete sterilization, although the authors did not examine the effect on mycoplasmas. This prompted us to study the effect of two ofloxacin concentrations on *M. bovis*. Contrary to expectations, even the high concentration of 400 µg/mL did not have a bactericidal effect on the reference strain. Antimicrobial resistance does not explain our results, as the MIC values of the tested strains were well below the concentrations of antibiotics used in semen extenders. In our study, extended semen was kept for 3 to 3.5 hrs at temperatures decreasing from 34 °C to 17 °C, thus allowing the antibiotics ample time to destroy the mycoplasmas. However, the biological conditions used in AI semen straw production differ markedly from the conditions in MIC testing, which could explain the difference seen between the MIC value of an antibiotic and its effect in semen on *M. bovis*.

We conclude that the effect of antibiotics on *M. bovis* in semen production depends on the contamination level. When there is a low level of contamination, GTLS used at high concentrations (500, 100, 300, and 600 µg/mL) is more efficient than GTLS used at the concentrations stated in the EU and OIE Terrestrial Code regulations. Our study also confirmed previous findings that *M. bovis* survives well in semen stored in liquid nitrogen.

6.6 DNA extraction from semen to detect *M. bovis* (IV)

One way to ensure *M. bovis*-free semen for AI could be culture or PCR testing of either raw or extended semen. However, mycoplasma culture is not a feasible method for use in the AI industry, because it requires specific media and is time consuming. Moreover, culture cannot directly discriminate between different

mycoplasma species potentially growing from semen samples. Only a few studies have examined the PCR method to detect *M. bovis* in semen [215,336]. Spermatocytes are rich in DNA, and semen has a high protein concentration and also contains zinc, all of which makes it challenging to extract good quality DNA from semen [215,337]. McDonald [336] and Parker et al. [215] used a DNA extraction method in which semen was first diluted in 2% Triton X buffer. According to McDonald [336], this reduces the adherence of *M. bovis* to the disulfide bond-rich acrosome membrane. DNA from Triton X-treated semen was extracted using a commercial extraction kit. To detect *M. bovis* in extracted DNA, McDonald [336] developed two rtPCRs targeting the housekeeping genes *fusA* and *oppD/F,* whereas Parker et al. [215] used primers and a probe against the *uvrC* gene. The limit of detection of the PCRs developed by McDonald [336] was 3.1 x 10^3 CFU/mL compared to 1.3 x 10^5 CFU/ml in the rtPCR of Parker et al. [215]. Vähänikkilä et al. [338] reported results from an interlaboratory trial to detect *M. bovis* in semen. A magnetic bead method was used to extract DNA. The limit of detection varied between 7.2 x 10^1 and 3.4 x 10^3 CFU/mL, depending on the PCR method used and the examining laboratory.

The InstaGene™ matrix is made of a specially formulated Chelex resin, which binds many PCR inhibitors. This method does not involve the use of organic solvents or alcohol precipitation, making it a simple and rapid method to isolate DNA. Chelex-based DNA extraction is used in forensic studies [339] and is described in the OIE Terrestrial Manual as a method to isolate herpesvirus DNA from bovine semen (chapter 3.4.11). We did not determine the limit of detection of *M. bovis* from DNA extracted from semen, as we used only two spiking concentrations. However, in our experience, the InstaGene™ method was the most sensitive one, as it detected *M. bovis* in 17 out of 18 studied semen straw pools compared to 12/18 and 10/18, respectively, when a commercial DNA extraction kit or the method of Parker et al. [215] was used and a low spiking concentration had been tested. Hence, further studies are warranted on use of the InstaGene™ method to extract *M. bovis* DNA from extended semen in AI industry.

6.7 Limitations and strengths of the studies

Regarding antibody testing, we did not attempt to sample the same animals repeatedly. If the same heifers and cows had been followed during the 2-year study period, we could have more precisely analyzed the antibody dynamics in herds with different infection statuses. However, this would have meant a larger number than 15 blood samples per age group, because some animals are always lost during follow-up studies. We did not sample calves under three months of age, so our study did not give any answer to question regarding the presence of maternal antibodies to *M. bovis*. Different time frames from the detection of the

index case and first BTM sample sent by the farmer to serological testing, besides the fact that not all farmers sent monthly BTM samples to testing, hindered us in properly analyzing the use of the Bio K 260 ELISA to detect antibodies in BTM.

We would have preferred to repeat the efficacy studies for some antibiotics against *M. bovis* in semen production. However, this was not possible, as we no longer have an AI station in Finland.

Semen used in AI has been suspected to be able to transmit *M. bovis* infection, but no actual evidence has been published. Because *M. bovis* had only recently been detected in Finland and was not widely spread among the cattle population, it gave us a good starting point to conduct the epidemiological study and provide evidence that commercial AI semen can indeed be a source of infection.

6.8 Future prospects

The inter-laboratory performance of a new commercial ELISA test, ID Screen® Mycoplasma bovis (IDVet, Grabels, France), has been evaluated against Western blotting and the Bio K302 ELISA [340]. The Se of the IDVet ELISA was 93.5% and Sp was 98.6% compared to respective figures of 91.8% and 99.6% for WB and 49.1% and 89.6% for BIO K302. Similarly, Petersen et al. [333] found that the IDVet ELISA is much more sensitive than Bio K302, but they questioned the specificity of the IDVet test, as cows in two herds with no history of *M. bovis*-associated disease also tested positive. Interestingly, they discovered a good correlation between serum and milk antibodies, indicating that composite milk samples could replace blood sampling. The use of the IDVet ELISA test in the Finnish *M. bovis* control program should be evaluated. This would require a study in which blood and milk samples from cows and primiparous cows, as well as blood samples from young stock are tested using this ELISA. Ideally, we should aim to replace blood samples with composite milk samples in the control program so that milk samples taken for dairy herd improvement and an automatic warning system to send these milk samples to ELISA testing could be used. This would probably make the control program more attractive to dairy farmers and more herds would join the program. Further studies to evaluate the use of blood and/or milk samples in herds in which *M. bovis* is only detected in young stock are also needed.

Areas or closed dairy and suckling cow herds free of *M. bovis* would be highly likely to benefit if they could buy semen certified to be free of *M. bovis*. Hence, cost-effective analytical methods should be developed for use in such a semen certification system.

7 CONCLUSIONS

1. No large outbreaks of mastitis were observed on Finnish dairy farms. The highest prevalence of *M. bovis* mastitis was 7.7%, and most farms had a prevalence of under 1%. On some farms, *M. bovis* was found to colonize young stock without causing mastitis.

2. The in-house MilA ELISA from serum was found to be more sensitive than the commercial Bio-X Bio K 260 ELISA, but further studies are needed to explore the specificity of the MilA ELISA. In a naive herd, *M. bovis* infection appears to rapidly spread among all age groups. The proportion of MilA antibody-positive cows remained high (80% to 100%) in all studied herds for at least one and half years after introduction of the infection into the herd.

3. RtPCR detection of *M. bovis* in BTM is not a cost-efficient method for use in *M. bovis* surveillance on Finnish dairy farms. The Bio K 260 ELISA detected antibodies in BTM on some farms three weeks to three months after the index mastitis case, whereas only one BTM sample was positive in the MilA ELISA.

4. *M. bovis*-contaminated commercial AI semen can introduce the infection into naive dairy herds. In a naturally infected bull, shedding of *M. bovis* into semen was intermittent and short-lived, and bacteria were not evenly distributed between the semen lots. Several straws, even from the same lot, must be examined to find contamination.

5. On recently infected dairy farms, calves shed *M. bovis* into nasal secretions, and a proportion of young calves are more often colonized with *M. bovis* in the nasopharynx than shedding the bacteria into nasal secretions. However, because NS samples are easily collected and more affordable compared to NP samples, thus allowing more samples to be taken, the inclusion of NS sampling of calves under six months of age is recommended in a control program.

6. NP sampling of calves with acute BRD has good agreement with more tedious and invasive BAL sampling. *M. bovis* can be reliably detected when 4 to 5 NP samples are taken from a group of calves.

7. The effect of antibiotics on the viability of *M. bovis* in extended semen depends on the level of contamination and concentration of antibiotics used. We recommend using the high GTLS concentration given in the CSS instructions. If the bacterial load is high (10^6 CFU/mL), even a high GTLS concentration will be ineffective.

8. Among the three tested DNA extraction methods, Instagene™ was the most sensitive DNA extraction method to detect *M. bovis* in semen using rtPCR.

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