

**EPIDEMIOLOGY OF *MYCOPLASMA BOVIS*  
INTRAMAMMARY INFECTION IN DAIRY HERDS**

*MYCOPLASMA BOVIS*E PÕHJUSTATUD  
UDARANAKKUSE EPIDEMIOLOOGIA  
PIIMAVEISEKARJADES

**ANRI AINO ELISA TIMONEN**

A Thesis  
applying for the degree of Doctor of Philosophy in Veterinary Science

Väitekirj  
filosoofiadoktori kraadi taotlemiseks loomaarstiteaduse erialal

**Eesti Maaülikooli doktoritööd**

**Doctoral Theses of the  
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*If you can dream it, you can do it.*

*- Walt Disney*

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## LIST OF ORIGINAL PUBLICATIONS

The thesis is based on three original publications (I-III). The articles are referred in the text using Roman numerical.

- I Timonen, A.A.E, Katholm, J., Petersen, A., Mõtus, K., Kalmus, P. 2017. Within-herd prevalence of intramammary infection caused by *Mycoplasma bovis* and associations between cow udder health, milk yield, and composition. *Journal of Dairy Science*, 100, 6554–6561.
- II Timonen, A.A.E, Autio, T., Pohjanvirta, T., Häkkinen, L., Katholm, J., Petersen, A., Mõtus K., Kalmus, P. 2020. Dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds. *Veterinary Microbiology*. Accepted 5 February 2020.
- III Timonen, A.A.E., Katholm, J., Petersen, A., Orro, T., Mõtus, K., Kalmus, P. 2018. Elimination of selected mastitis pathogens during the dry period. *Journal of Dairy Science*, 101, 9332–9338.

### The contribution of the authors to the research papers

Paper	Original idea, study design	Data collection	Data analysis	Preparation of manuscript
I	AT, KM, PK	AT, PK	AT, AP, KM	All authors
II	AT, KM, PK	AT, KM, PK	AT, LH, AP, KM, TA	All authors
III	AT, KM, PK	AT, PK	AT, AP, KM, TO	All authors

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## ABBREVIATIONS

APP	acute phase protein
APR	acute phase response
BRD	bovine respiratory disease
BTM	bulk tank milk
CFU	colony forming units
cg-MLST	core genome multilocus sequence typing
CI	confidence interval/ credibility interval
CMS	composite milk sample
DIM	days in milk
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
Hp	haptoglobin
IMI	intramammary infection
<i>M. bovis</i>	<i>Mycoplasma bovis</i>
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight mass spectrometry
MIC	minimal inhibitory concentration
ODC	optical density coefficient
PCR	polymerase chain reaction
rt-PCR	real-time polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SCC	somatic cell count
<i>Staph. aureus</i>	<i>Staphylococcus aureus</i>
<i>Strep. agalactiae</i>	<i>Streptococcus agalactiae</i>
<i>Strep. uberis</i>	<i>Streptococcus uberis</i>
TBL	tracheobronchial lavage
VSP	variable surface lipoprotein

# 1. INTRODUCTION

Mastitis, an inflammation of the mammary gland, is the most common disease affecting dairy cattle worldwide. *Mycoplasma* infections were first identified in naturally occurring mastitis cases in cattle in the 1960s (Jain *et al.*, 1969) and in calves with arthritis in the 1970s (Pfützner and Schimmel, 1979). *Mycoplasma (M.) bovis* is an emerging udder pathogen causing intramammary infection (IMI), which commonly leads to mastitis (Gonzalez *et al.*, 1992; Nicholas and Ayling, 2003). In addition to mastitis in dairy cows, *M. bovis* causes respiratory disease and arthritis in calves (Stalheim and Page, 1975; Nicholas *et al.*, 2002). *M. bovis* infection seems also to lower the weight gain of calves (Nicholas *et al.*, 2002), affecting calf welfare.

The effect of *M. bovis* infections to the cattle industry is negative both economically and considering animal welfare. *M. bovis*-associated IMI causes economic losses to the dairy industry due to lower milk yield and decreased milk quality (Al-Farha *et al.*, 2017). Compared to other major mastitis pathogens, lower 305-day mature equivalent milk production was detected in cows with *Mycoplasma* mastitis, leading to an estimated economic loss of \$451.63 per lactation (Wilson *et al.*, 1997). Additionally, somatic cell count (SCC) is usually higher in cows with *M. bovis* IMI (Fox, 2012), decreasing milk quality. Undesirably, *M. bovis*-seropositive heifers have higher risk for undesired early departure from the herd before first calving (Petersen *et al.*, 2019).

In recent decades, new molecular diagnostic methods have been established. The polymerase chain reaction (PCR) method has replaced the traditional bacteriological cultivation in diagnosis of bovine mastitis due to the high sensitivity and speediness of the test (Koskinen *et al.*, 2010; Hiitö *et al.*, 2015). Bacteriology of genus *Mycoplasma* is slow and has low sensitivity (González and Wilson, 2002; Fox *et al.*, 2005). Molecular diagnostic methods have made the diagnosis of *M. bovis* infections easier, probably increasing the prevalence of *M. bovis* IMI worldwide (Fox *et al.*, 2005). Rapid diagnosis of *M. bovis* IMI is essential to avoid the economic losses together with avoiding the spread of the disease within and between dairy herds.

In 2018, the Estonian dairy cow population was 85,200 dairy cows, of which 81,821 (96.0%) dairy cows belonged to a voluntary basis milk recording system. Of all dairy herds, 49.1% of the dairy herds had  $\geq 50$  cows in the herd. An average 305-day milk yield per cow was 9,785 kg in 2018 (Estonian Livestock Performance Recording Ltd., 2019). Although the proportion of large dairy herds is moderate, a relatively large proportion of the Estonian dairy cows (32.8%) are housed in herds with  $\geq 100$  dairy cows (Estonian Livestock Performance Recording Ltd., 2019). *M. bovis* IMI is more prevalent in large dairy herds (USDA-APHIS, 2008). Previously, no prevalence or epidemiologic studies have studied *M. bovis* IMI in Estonia. However, the Estonian dairy population serves as an adequate study population for endemic *M. bovis* IMI studies because the pathogen has been identified in Estonian dairy operations annually (Estonian Veterinary and Food laboratory, 2018), and no official control schemes are established for the disease.

Despite the long history and worldwide presence of *M. bovis* infections in cattle, the epidemiology of *M. bovis* infections in dairy herds is still not fully understood. Similarly, the within-herd prevalence of *M. bovis* IMI is not widely studied, although knowledge about the dynamics of the within-herd prevalence of *M. bovis* IMI would benefit the disease surveillance.

This dissertation focuses on the prevalence and epidemiology of *M. bovis* mastitis in Estonian dairy herds. In Study I, the within-herd prevalence of *M. bovis* IMI was evaluated in one dairy herd, and associations between *M. bovis* IMI and cow milk yield and milk quality were studied. In Study II, the *M. bovis* IMI within-herd prevalence was evaluated in four dairy herds with repeated cross-sectional study design. Additionally, cow clinical mastitis and calf respiratory isolates of *M. bovis* were characterised in Study II. The elimination of *M. bovis* IMI during the dry period and associations with milk haptoglobin concentration were evaluated in Study III.

## 2. REVIEW OF THE LITERATURE

### 2.1. An overview of the *Mycoplasma bovis* infections

#### 2.1.1. Characterisation of *Mycoplasma bovis*

*Mycoplasma spp.* cause bovine mycoplasmosis, leading to multiple types of infections in cattle (Bürki *et al.*, 2015). Several *Mycoplasma* species are associated with cattle infections, the most dominant species being *M. bovis* (Gioia *et al.*, 2016). Other less prevalent *Mycoplasma* species causing infections in cattle include for example *M. bovirhinalis*, *M. californicum*, *M. bovirhinalis*, *M. alkalescens* and *M. dispar* (Nicholas and Ayling, 2003; Fox, 2012; Gioia *et al.*, 2016).

*Mycoplasma* belong to the *Mollicute* genus bacteria, which lack a cell wall and are the smallest self-replicating bacteria with a parasitic way of life (Razin, 1997; Rosengarten *et al.*, 2000). *Mycoplasma* cells have three main cell organelles: the cell membrane, ribosomes, and circular DNA. The cells are spherical with a diameter of 0.3–0.8  $\mu\text{m}$  (Razin and Hayflick, 2010). Fatty acids and sterols as nutrients are necessary for *Mycoplasma* cells (Razin and Hayflick, 2010). Demanding growth requirements and the simple attribute of the *Mycoplasma* cells enables the adaptation of bacteria with the host organism (Fox, 2012). Sunlight and desiccation are injurious to *Mycoplasma* cells. Despite this, *M. bovis* may survive in cool and humid conditions for long periods (Pfützner, 1984), probably due to biofilm formation (McAuliffe *et al.*, 2006).

In general, pathogenic characteristics of *Mycoplasma* include adherence and internalization to host cells, immunomodulatory characteristics and ability to colonise host tissue (Maunsell *et al.*, 2011; Fox, 2012). All these pathogenic characteristics contribute to evade the host immune response and cause a chronic manifestation of *M. bovis* infection (Buchenau *et al.*, 2010; Fox, 2012). Additionally, biofilm formation by *M. bovis* enables the pathogen to survive in the environment and inside the host (McAuliffe *et al.*, 2006).

During *Mycoplasma* infection, adhesion to host cells is usually the first phenomenon (Rottem, 2003). Adhesion is necessary for successful

colonisation, which is driven by avoidance of physical removal through mucociliary clearance, and for acquisition of nutrients (Rosengarten *et al.*, 2000). Membrane proteins of the *Mycoplasma* cell form the main interface with the host and enable the adherence to host cells (Sachse *et al.*, 1996; Lysnyansky *et al.*, 1999; Buchenau *et al.*, 2010). Variable surface lipoproteins (VSP) and other types of adhesions are involved in the adherence of *M. bovis* to the host cells (Sachse *et al.*, 1996; Thomas *et al.*, 2003; Fox, 2012). The adherence of *M. bovis* to host cells may depend on the strain of the pathogen. Virulent *M. bovis* strains have higher adherence rates compared to less or non-virulent strains (Thomas *et al.*, 2003). After adherence, *M. bovis* produces phospholipases, hydrogen peroxide and superoxide radicals, damaging the host cells (Khan *et al.*, 2005; Bürki *et al.*, 2015).

### **2.1.2. Host immune response in *Mycoplasma bovis* infections**

VSP on *Mycoplasma* cell membrane are produced after exposure to host antibodies being therefore antibody-specific and providing a large antigenic variation (Buchenau *et al.*, 2010; Browning *et al.*, 2011). There are multiple genes coding the VSP (Lysnyansky *et al.*, 1996, 1999) and several VSP may coexist on the cell membrane leading to specific structural and antigenic features of *M. bovis* (Lysnyansky *et al.*, 1999; Bürki *et al.*, 2015). Hence, the variation in antigenic heterogeneity of *M. bovis* is independent of the geographical origin and type of organ or disease produced by a single strain (Rosengarten *et al.*, 1994).

Host cell invasion after adherence allows *Mycoplasma* to evade the host immune response (Rosengarten *et al.*, 2000). *M. bovis* has been detected from peripheral blood mononuclear cells, erythrocytes (van der Merwe *et al.*, 2010) and macrophages and neutrophils, whereas *M. bovis* antigen has been detected in lymph nodes and occasionally from bronchiolar epithelial cells (Adegboye *et al.*, 1995; Rodriguez *et al.*, 1996; Kleinschmidt *et al.*, 2013). However, *M. bovis* has not been detected in alveolar epithelial cells of the mammary gland (Stanarius *et al.*, 1981, reviewed by Bürki *et al.*, 2015). Intracellular persistence of *M. bovis* in phagocytes is presumably attributed to an alteration of the process of phagocytosis after engulfment (Kleinschmidt *et al.*, 2013). Along with avoiding the host immune response, invading host cells enable *M. bovis* to reach multiple organ systems and body sites of the diseased animal, cause chronic infections and impair the antibiotic treatment of *M. bovis*

infections (Rosengarten *et al.*, 2000; Bürki *et al.*, 2015). Jain *et al.* (1969) identified *Mycoplasma* from blood of two cows experimentally infected with *Mycoplasma* originating from clinical mastitis cases.

The modulation of the host's immune response is operated mainly through altered immune cell and cytokine functions (Bürki *et al.*, 2015) leading to prolonged survival and systemic dissemination of *M. bovis* in host organism (Mulongo *et al.*, 2014). Both immunosuppression and stimulation are described. Stimulation of the immune system is suspected to occur through macrophages, T cells or complement activation together with the presence of cytokines, leading to upregulated immune response (Vanden Bush and Rosenbusch, 2003; Kauf *et al.*, 2007). On the contrary, expression of anti-inflammatory cytokines and chemokines, such as interleukin (IL)-10, together with suppression of pro-inflammatory cytokines, such as interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$ , production are considered the reasons for immunosuppression (Mulongo *et al.*, 2014). Other explanations for immunosuppression might be a downregulation of lymphocyte proliferation, formation of putative mycoplasmal lympho-inhibitory protein or interference of lympho-proliferative response to phytoagglutinin (Thomas *et al.*, 1990; Vanden Bush and Rosenbusch, 2003; van der Merwe *et al.*, 2010). Studies about *M. bovis*-induced apoptosis of peripheral blood mononuclear cells are controversial. Some studies describe induction of apoptosis in lymphocytes by *M. bovis* *in vitro* (Vanden Bush and Rosenbusch, 2002), and others result in delayed apoptosis in bovine monocytes infected with *M. bovis* (Mulongo *et al.*, 2014). Additionally, *M. bovis* might obtund the host immune response by binding to neutrophils and inhibiting their oxidative burst (Thomas *et al.*, 1991) and suppress immune response in bovine mammary epithelial cells (Gondaira *et al.*, 2018).

After injury, acute phase response (APR) is triggered by cytokines, messenger proteins between active site of injury and hepatocytes. During APR, serum concentrations of acute phase proteins (APP), which are mainly produced in hepatocytes, change intrinsically (Petersen *et al.*, 2004). In addition to injuries, both inflammatory and infectious processes may trigger the APR, increasing concentrations of APP in serum (Hiss *et al.*, 2004; Thielen *et al.*, 2007). Bovine haptoglobin (Hp) and serum amyloid A (SAA) are APPs serving as non-specific indicators of inflammatory response (Petersen *et al.*, 2004). Although APR is commonly thought to be part of a general defence mechanism toward

tissue injury (Petersen *et al.*, 2004), stress is also associated with increased serum APP concentrations (Alsemgeest *et al.*, 1995). Hypothetically, serum APP levels can be used as markers of herd health (Gånheim *et al.*, 2007) and sub-clinical inflammation in cattle (Karreman *et al.*, 2000). An increase in serum concentration of APP is associated with both viral respiratory pathogens, such as bovine respiratory syncytial virus (Heegaard *et al.*, 2000; Orro *et al.*, 2011) and bovine virus diarrhoea virus (Gånheim *et al.*, 2003) and bacterial pathogens, including *P. multocida* (Nikunen *et al.*, 2007) and *M. haemolytica* (Gånheim *et al.*, 2003) in naturally occurring respiratory infections and experimental trials. Additionally, local production of Hp in mammary glands occurs during IMI as Hp is released from the damaged epithelial cells and neutrophils (Hiss *et al.*, 2004). However, associations between *M. bovis* respiratory disease or IMI and serum or milk APP concentrations have not been previously studied.

Natural *M. bovis* infections, such as mastitis, arthritis and pneumonia, alter antibody response in the organism (Hazelton *et al.*, 2018a; Petersen *et al.*, 2018). The seroconversion is thought to take up to one month (Foster *et al.*, 2009), while vaccination causing seroconversion occurs in two to three weeks (Nicholas *et al.*, 2002). However, the duration of elevated antibody titres after natural exposure to *M. bovis* has not been well characterised, even though Byrne *et al.* (2000) suspected that antibodies stay elevated for several months after *M. bovis* outbreak. Both Hazelton *et al.* (2018a) and Petersen *et al.* (2018) state that antibody response after *M. bovis* infection may differ between individual cows, with some seroconverting and others not. *M. bovis* mastitis causes elevated antibody concentrations in milk and serum (Petersen *et al.*, 2018). On the contrary, antibody levels in milk in cows having *M. bovis* infections other than mastitis are not elevated (Petersen *et al.*, 2018). Hence, the antibody response in milk after *M. bovis* mastitis is probably local (Byrne *et al.*, 2000).

### **2.1.3. *Mycoplasma bovis*-associated diseases**

Mycoplasmoses are slowly progressive chronic infections (Rosengarten *et al.*, 2000). *M. bovis* causes most often IMI in adult cattle, leading to subclinical or clinical mastitis (González and Wilson, 2003) and bovine respiratory disease (BRD) and arthritis in cattle of all ages (Stalheim and Page, 1975; Nicholas *et al.*, 2002). Additionally, *M. bovis* may occasionally

cause abortions (Doig, 1981), meningitis (Stipkovits *et al.*, 1993), otitis media (Francoz *et al.*, 2004; Foster *et al.*, 2009), keratoconjunctivitis (Alberti *et al.*, 2006) and subcutaneous abscesses (Kinde *et al.*, 1993). Pathogens have also been identified from post-surgical seromas (Gille *et al.*, 2016).

The course of *M. bovis* mastitis in dairy herds is usually endemic subclinical disease, although severe clinical mastitis outbreaks may also occur (Pothmann *et al.*, 2015). *M. bovis* IMI may occur without elevated milk somatic cell count (SCC) (Pinho *et al.*, 2013). Pinho *et al.* (2013) found that in three cows having negative California Mastitis test result, udder quarter milk samples (n = 4) were *M. bovis*-positive. In a study made by Kauf *et al.* (2007), SCC increased 66 hours after infection, and during a ten-day study period, SCC stayed elevated in dairy cows experimentally infected with *M. bovis*. The maximum SCC in milk was 120 million cells/ml (Kauf *et al.*, 2007). Clinical *M. bovis* mastitis does not induce specific clinical signs. In general, several udder quarters are infected, milk yield decreases dramatically, the mammary gland might be swollen, and milk secretions might be mildly abnormal to purulent with brownish colour. Severe systemic signs are usually absent as febrile response is not induced by *M. bovis* IMI (Kauf *et al.*, 2007). Clinical *M. bovis* mastitis may persist for several weeks. Mastitis caused by *M. bovis* is also detected in dry cows (Otter *et al.*, 2015).

*M. bovis* may occur in BRD complex, usually in calves (Rosendal and Martin, 1986; Schibrowski *et al.*, 2018). In naturally infected cattle with pneumonia, *M. bovis* is commonly detected with other microorganisms (Shahriar *et al.*, 2002; Booker *et al.*, 2008), mainly with bacterial pathogens *Mannheimia haemolytica*, *Histophilus somni* and *Pasteurella multocida* (Byrne *et al.*, 2001; Griffin *et al.*, 2010). Natural *M. bovis* pneumonia is characterised with exudative bronchopneumonia with possible coagulative necrosis (Rodriguez *et al.*, 1996). Radaelli *et al.* (2008) associated a high number of intralesional organisms with a severe necrosuppurative bronchopneumonia or fibrinonecrotising pneumonia, whereas a low number of organisms was associated with mild catarrhal bronchointerstitial pneumonia. Clinical signs in *M. bovis*-induced respiratory disease are non-specific, including fever, tachypnoea, dyspnoea and decreased appetite. Coughing and nasal discharge may also occur (Stipkovits *et al.*, 2000). Clinical signs may be more severe if coinfection of *M. bovis* and other respiratory pathogens exists.

Both *M. bovis* mastitis and respiratory disease may occur together with other manifestations of disease. Arthritis and respiratory disease may occur together with *M. bovis* mastitis (Pothmann *et al.*, 2015). Furthermore, *M. bovis* pneumonia might be accompanied by otitis media, arthritis or both (Mahmood *et al.*, 2017).

#### 2.1.4. Transmission of *Mycoplasma bovis* infections

Introduction of *M. bovis* infection to the dairy herd is usually conducted by importing subclinically infected animals to the herd (Fox *et al.* 2003; Gonzalez and Wilson, 2003; Filioussis *et al.* 2007; Fox 2012). More recently, contaminated semen used for artificial insemination has been proven to be a source of *M. bovis* infection to dairy herds in Finnish study by Haapala *et al.* (2018). Additionally, Hazelton *et al.* (2018b) identified an increase from 9 to 46% in the seroprevalence of *M. bovis* among bulls introduced to *M. bovis*-positive dairy herds for the breeding period. Four bulls had *M. bovis* culture-positive semen samples (Hazelton *et al.*, 2018b). Researchers concluded that infected bulls could be potential transmitters of *M. bovis* infection into a naïve dairy herd (Hazelton *et al.*, 2018b).

*M. bovis* is considered to belong to a group of contagious mastitis pathogens together with *Staphylococcus (Staph.) aureus* and *Streptococcus (Strep.) agalactiae* (Royster and Wagner, 2015). Contagious udder pathogens can attach to the udder epithelia (Frost *et al.* 1977; Royster and Wagner, 2015). Cows with *M. bovis* IMI shed bacteria with milk (Jasper, 1977). Hence, transmission of *M. bovis* IMI between diseased and healthy cows may occur during milking (Ruegg and Erskine, 2015). Milking machine, hands of the milk maid and cleaning towels may all serve as vectors for transmission of bacteria, especially if cows with mastitis are milked together with healthy cows (Punyapornwithaya *et al.*, 2011; Keefe, 2012).

As animals also excrete *M. bovis* with other secretions, transmission of pathogens can also occur through contaminated feed, water or bedding material (Fox *et al.*, 2005; Justice-Allen *et al.*, 2010; Maunsell *et al.*, 2011). Transmission between cows and calves is commonly thought to occur via contaminated milk (Butler *et al.*, 2000). *M. bovis* has also been isolated from vaginal secretions of cows at calving (Feenstra *et al.*, 1991) and from foetuses and placenta (Hassan and Dokhan, 2004; Hermeyer *et al.*, 2012). Hence, vertical transmission of *M. bovis* infection may also occur.

### 2.1.5. Diagnosis of *Mycoplasma bovis* infections

Traditionally, microbiological methods are used to identify bacteria from pathologic material, such as milk or respiratory secretions, by cultivating material on specific agar plates. On-farm culture methods are developed to detect udder pathogens rapidly and with high sensitivity (Lago *et al.*, 2011; Cameron *et al.*, 2014). However, *Mycoplasma* cannot be cultivated with traditional agar plates detecting other common pathogens. A modified Hayflick agar plate is needed for cultivation of *Mycoplasma* (Dorman *et al.*, 1983). Plates are incubated for seven to ten days under environmental conditions of 35 °C and 10% CO<sub>2</sub> concentration for culture growth (Dorman *et al.*, 1983; Maunsell *et al.*, 2011; Ruegg and Erskine, 2015). A long incubation period is non-economic and delays the diagnosis (Fox *et al.*, 2005; Boonyayatra *et al.*, 2012). Additionally, cultivation of *Mycoplasma* has low accuracy, probably due to the extreme nutrient demands and difficulties of preventing other bacteria from growing on the Hayflick plates (Gonzalez and Wilson, 2003; Maunsell *et al.*, 2011). Low bacterial concentration due to intermittent shedding of *M. bovis* may cause false-negative cultivation results (González and Wilson, 2002). In a study by Biddle *et al.* (2003), cows with *Mycoplasma* mastitis shed bacteria in different amounts. Only 39% of the cows shed *Mycoplasma* with milk in an amount of  $\geq 100$  CFU/ml. Furthermore, freezing of samples for storage may influence the recovery of *Mycoplasma*, leading to no growth in cultivation (Biddle *et al.*, 2004; Boonyayatra *et al.*, 2010; Olde Riekerink *et al.*, 2010; Ruegg and Erskine, 2015). However, freezer storage time and number of freeze-thaw cycles influences the recovery of *Mycoplasma* during freezing. Single and double freeze-thaw cycles have reduced the *M. bovis* concentration by 1 and 1.5 log in colostrum, while 14-week storage time at -18 °C **did not significantly** decrease the survival of *M. bovis* compared to one-week storage time (Gille *et al.*, 2018a). Bacterial cultivation detects only the genus *Mycoplasma*. Further identification of *Mycoplasma* species should be done with molecular methods (Fox *et al.*, 2005).

Development of matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) has emerged as a new, rapid and accurate technology for species and specific bacterial characteristic identification from bacterial colonies (Firacative *et al.*, 2012; Pereyre *et al.*, 2013; Lasch *et al.*, 2015). *Mycoplasma* VSP are detected with MALDI-TOF (Vanden Bush and Rosenbusch, 2004; Sun *et al.*, 2014).

Additionally, Pereyre *et al.* (2013) correctly identified 95 to 98% of ruminant *Mycoplasma* species and were able to differentiate *M. bovis* from *M. agalactiae* using MALDI-TOF. However, only bacteria belonging to the reference database can be identified using MALDI-TOF.

The polymerase chain reaction (PCR) method is commonly used in pathogen detection. Advantages of the PCR method are the sensitivity and fast analysis of the pathogenic material together with the ability of the method to depict bacteria at species level (Hiitiö *et al.*, 2015). Commercial test kits, such as PathoProof PCR assays (ThermoFisher Scientific) or Mastitis 4 (DNA Diagnostic A/S), include DNA detection of genus *Mycoplasma* and different *Mycoplasma* species, depending on the test kit. Milk or respiratory secretions can be analysed for *M. bovis* with PCR without previous cultivation of bacteria (Fox *et al.*, 2005; Cornelissen *et al.*, 2017). Boonyayatra *et al.* (2012) identified an overall accuracy of 98.51% for the real-time PCR (rt-PCR) method for *Mycoplasma* analysing milk and organ tissues. They also identified *Mycoplasma* with rt-PCR directly from milk with bacterial concentrations of 10–30 CFU/ml (Boonyayatra *et al.*, 2012). Previously, DNA of *Mycoplasma* has been detected with rt-PCR from milk with bacterial concentration of  $\leq 100$  CFU/ml (Sachse *et al.*, 2010). The sensitivity of the PCR method depends on sample type and concentration of bacterial DNA in the sample (de Carvalho *et al.*, 2015). Additionally, highly sensitive PCR also detects DNA of bacteria already killed by the host organism, which may cause clinically irrelevant treatment decisions to be made (Fox *et al.*, 2005; Hiitiö *et al.*, 2015) and only DNA of bacteria included to the test kit are detected (Hiitiö *et al.*, 2015).

Antibodies against *Mycoplasma* can be detected by the enzyme-linked immunosorbent assay (ELISA) method. *Mycoplasma* antibody concentration can be measured from both milk and serum (Thomas *et al.*, 1987; Byrne *et al.*, 2000). A high specificity for detection of *M. bovis* antibodies is described (Byrne *et al.*, 2000). Even though there are multiple pathogenic *Mycoplasma* species, ELISA test cannot distinguish between different species (Fox *et al.*, 2005).

Both herd- and animal-level diagnosis are used in diagnostic protocols to detect *M. bovis* infections in dairy herds. For herd-level diagnosis and surveillance of *M. bovis*, bulk milk testing is usually performed. *M. bovis* antibodies (Nielsen *et al.*, 2015; Petersen *et al.*, 2016; Parker *et al.*, 2017),

DNA of *M. bovis* (Pinho *et al.*, 2013; Bauman *et al.*, 2018) or viable bacteria (Olde Riekerink *et al.*, 2006; McDonald *et al.*, 2009) can be identified from bulk milk, indicating the presence or previous exposure of *M. bovis* in the herd. In general, a positive bulk milk culture for *Mycoplasma* is thought to indicate at least one cow shedding the bacteria (Biddle *et al.*, 2003). Both bulk milk ELISA and PCR are sensitive to identify *M. bovis*-positive dairy herds, especially when focusing on positivity of dairy cows. Nielsen *et al.* (2015) identified a sensitivity of 43.5% and specificity of 99.6% of bulk milk ELISA for detection of *M. bovis* antibodies using the cut-off value 50% optical density coefficient (ODC%). Petersen *et al.* (2016) associated a higher cow seroprevalence of *M. bovis* with increased bulk milk ODC%, while the seroprevalence of *M. bovis*-positive youngstock did not correlate with the bulk milk ODC%. Parker *et al.* (2017) were able to detect *M. bovis* antibodies from bulk milk zero to eight months after *M. bovis* outbreak in herds. A sensitivity of 76.7% of PCR analysis of *Mycoplasma* from bulk milk was identified in a study by Justice-Allen *et al.* (2011). Additionally, Parker *et al.* (2017b) found that bulk milk samples had more samples identified as positive by multiplex probe PCR (n = 11) than by culture (n = 4).

*M. bovis*-positive bulk milk indicates only presence of the pathogen in the dairy herd. The within-herd prevalence of *M. bovis* IMI cannot be estimated based on positive bulk milk, and diseased animals should be identified by testing (Fox *et al.*, 2005) either individually or by groups. Separate testing should be performed to identify the presence and circulation of *M. bovis* among youngstock.

Estimation of the within-herd prevalence of *M. bovis* IMI is complicated (Fox, 2012), and it is usually evaluated by analysing cow composite milk samples (CMS) (Ghadersohi *et al.*, 1999; Filioussis *et al.*, 2007; Murai *et al.*, 2014). Murai *et al.* (2014) discovered the most cost-effective strategy to identify *M. bovis* IMI-positive dairy cows to be two-staged culture strategy, in which pools of five cows' CMS were first cultured, and then individual cow CMS that constituted positive pools was cultured. Sensitivity of the strategy was 73.5% (Murai *et al.*, 2014). Cow CMS or udder quarter milk samples may also be analysed with PCR to identify *M. bovis*-positive cows or quarters.

Serological testing of youngstock may be used to detect calves and heifers positive to *M. bovis* antibodies (Petersen *et al.*, 2016; Petersen *et*

*al.*, 2019). Additionally, PCR analysis from bronchoalveolar lavage fluid may be used to identify *Mycoplasma* species from BRD cases (Cornelissen *et al.*, 2017).

## 2.1.6. Prevalence of *Mycoplasma bovis*

### 2.1.6.1. Prevalence of *Mycoplasma bovis* in milk

The exposure to *M. bovis*-associated diseases is studied by identifying *M. bovis* antibodies from serum and milk both at individual animal and herd level. Identification of herd prevalence of *M. bovis* is usually made by bulk tank surveys (Fox, 2012). Prevalence of *M. bovis* in bulk tank milk surveys has fluctuated between zero and 62.4% (Table 1). Bulk milk analysis of *M. bovis* antibodies revealed that among all Danish dairy herds ( $n = 3,700$ ), 1.5 to 5.2% were antibody-positive for *M. bovis* in bulk tank milk during a one-year study period between June 2013 and July 2014 in four consecutive samplings (Arede *et al.*, 2016). Nielsen *et al.* (2015) identified a 7.2% prevalence of *M. bovis* antibodies in bulk tank milk of 3,437 Danish dairy herds. In Belgium, 17% of the randomly selected hundred bulk milks tested were antibody-positive for *M. bovis* (Gille *et al.*, 2018b).

**Table 1.** Prevalence of *M. bovis* in bulk tank milk in different countries

	Tested herds ( <i>n</i> )	Positive herds ( <i>n</i> )	Positive herds (%)	Diagnostic method	Study year	Reference
<b>Australia</b>						
	238	1	0.4	PCR	2012	Penry <i>et al.</i> 2012
North-Queensland	186	81	43.5	MB-PCR <sup>1</sup>	1996	Ghadersohi <i>et al.</i> 1999
Victoria	165	103	62.4	MB-PCR	1996	Ghadersohi <i>et al.</i> 1999
<b>New Zealand</b>						
	244	0	0.0	PCR, culture	2007	McDonald <i>et al.</i> 2009
<b>Europe</b>						
Belgium	100	7	7.0	PCR	2016	Gille <i>et al.</i> 2018b
Portugal	164	4	2.4	PCR	2007-2008	Pinho <i>et al.</i> 2013

	Tested herds ( <i>n</i> )	Positive herds ( <i>n</i> )	Positive herds (%)	Diagnostic method	Study year	Reference
Belgium	200	3	1.5	PCR	2009	Passchyn <i>et al.</i> 2012
France	345	0	0.0	PCR	2005	Arcangioli <i>et al.</i> 2011
Greece	37	2	5.4	PCR	1997-1999	Filioussis <i>et al.</i> 2007
<b>North-America</b>						
Canada	372	2	0.5	PCR	2015	Bauman <i>et al.</i> 2018
Canada	258	4	1.6	Culture	2004	Olde Riekerink <i>et al.</i> 2006
USA	534	17	3.2 <sup>2</sup>	Culture	2007	USDA-APHIS 2008
USA	243	73	30.0	Culture <sup>3</sup>	NA <sup>4</sup>	Kirk <i>et al.</i> 1997
USA, New York state	561	7	1.2	Culture	1989-1990	Gonzalez <i>et al.</i> 1992
USA, Vermont	2,346	3	0.13 <sup>5</sup>	Culture	1983-1984	Kunkel 1985

<sup>1</sup> MB-PCR- Methyl-binding polymerase chain reaction

<sup>2</sup> *Mycoplasma* spp.

<sup>3</sup> >100 colonies/plate

<sup>4</sup> Not available

<sup>5</sup> The infection rate of 1.3 herds per thousand

*Mycoplasma* was identified from bovine mastitis cases already in 1960s (Jain *et al.*, 1969). During recent years, the prevalence of *Mycoplasma* mastitis has increased (Fox *et al.*, 2005). A higher prevalence of *Mycoplasma* mastitis is associated with larger herd size (USDA-APHIS, 2008). In a study from the US, the prevalence of *Mycoplasma* in bulk tank among herds with more than 500 dairy cows was 14.4% compared to prevalence of 1.8% among herds with fewer than 100 dairy cows (USDA-APHIS, 2008).

*M. bovis* has also been identified from clinical mastitis cases even though the prevalence varies largely between studies. In one Italian herd, 32 (n of tested cows = 122), cows had clinical signs due to *M. bovis* mastitis evaluated by milk samples of pooled quarters (Radaelli *et al.*, 2011). In

a study by Filioussis *et al.* (2007), 219 udder quarter milk samples were collected from cows with clinical mastitis, with 18 (8.2%) udder quarter milk samples being positive for *M. bovis*. Arcangioli *et al.* (2011) estimated a prevalence of <0.44% with 95% confidence interval (CI) in clinical mastitis cases based on 828 samples of mastitic milk.

The within-herd prevalence of *Mycoplasma bovis* intramammary infection has not been widely studied. Murai *et al.* (2014) identified a 2.8% (95% CI 1.9; 3.7) (n = 1210) within-herd prevalence of *Mycoplasma* IMI based on cow CMS PCR analyses. Filioussis *et al.* (2007) analysed cow CMS from 111 clinically healthy cows without identifying *M. bovis* from any of the samples.

#### **2.1.6.2. Seroprevalence and prevalence of respiratory manifestation of *Mycoplasma bovis***

The seroprevalence of *M. bovis* antibodies in individual animals varies widely depending on the study region and is described to be 82.91% (n = 860) in a Hungarian study including 86 cattle farms (Fodor *et al.*, 2017) and 76.7% (n = 3,670) in a Polish study with 361 herds (Bednarek *et al.*, 2012). Hanzlicek *et al.* (2011) observed *M. bovis* antibodies in a longitudinal study in the US during a 42-day study period, identifying *M. bovis* seroprevalence of 26.6% at day 0 and 98.2% at day 42 in stocker calves, respectively. On the contrary, Gulliksen *et al.* (2009) identified a 0% seroprevalence for *M. bovis* during a one-year study in 1,348 calves from 135 dairy herds in Norway. Similarly, in a Finnish study by Härtel *et al.* (2004), antibodies against *M. bovis* were not identified in calves with respiratory disease, even though antibodies for another *Mycoplasma spp.* were detected. Ayling *et al.* (2004) described a seroprevalence of 22% (n = 8,959) of *M. bovis* antibodies mainly in pneumonic cattle in Britain.

*M. bovis*-associated respiratory disease is one of the most common manifestations of mycoplasmosis, especially in calves. Francoz *et al.* (2015) evaluated the prevalence of *M. bovis* in 11 dairy herds with high incidence of BRD and detected *M. bovis* in 19 calves (n = 95) with PCR or culture from nasal swab and nasopharyngeal swab samples. Additionally, Moore *et al.* (2015) identified a *M. bovis* prevalence of 4.8% (n = 1,484) in clinically healthy cattle nasal swab samples with PCR. At post-mortem examination, Bell and co-workers (2014) identified *M. bovis* from 53 cattle (35.3%) with PCR and from 29 cattle (19.3%) by culture.

Castillo-Alcala *et al.* (2012) studied the prevalence of *M. bovis* at multiple times after arrival of cattle to feedlot. They found that prevalence of *M. bovis* in cattle bronchoalveolar lavage fluid was 1.7% (n = 60) at arrival, 72.2% (n = 36) at <15 days after arrival and 85.7% (n = 42) at 55 days after arrival. *M. bovis* alone was also detected by culture from 41.1% (n = 90) of calves' lung lesions at slaughterhouse (Soehnlén *et al.*, 2012). On the other hand, Bottinelli *et al.* (2017) found that 85.7% (n = 49) of nasal swab samples from veal calves were positive for *Mycoplasma spp.* with PCR but none of the samples were PCR positive for *M. bovis*.

### 2.1.7. Treatment of *Mycoplasma bovis* infections

*M. bovis* mastitis has been reported to be non-responded to systemic or intramammary antibiotic therapy as early as the 1970s, and treatment of cows with *M. bovis* mastitis is not recommended (Maunsell *et al.*, 2011). Dry cow therapy with antibiotics is also not effective against *M. bovis* IMI (Ruegg and Erskine, 2015).

*Mycoplasma* lack a cell wall, leading to resistance against antibiotics affecting cell wall of bacteria. Hence,  $\beta$ -lactam antimicrobials (Fox, 2012) or glycopeptides (Gautier-Bouchardon *et al.*, 2014) are not effective in the treatment of *Mycoplasma* infections. Additionally, *Mycoplasma* does not synthesise folic acid, causing resistance against sulphonamides (Rosenbusch *et al.*, 2005). Antimicrobials interfering with microbial proteins, such as tetracyclines or macrolides, and nucleic acid synthesis, like fluoroquinolones, are in general more effective against *Mycoplasma* (Francoz *et al.*, 2005). However, resistance against the macrolide group antimicrobial erythromycin may occur (Rosenbusch *et al.*, 2005). Susceptibility testing of antimicrobials for *Mycoplasma* in animals is not currently standardised, and no official minimum inhibitory concentrations (MIC) for *Mycoplasma* isolated from animals are available (Maunsell *et al.*, 2011). Therefore, the interpretation of *Mycoplasma* susceptibility testing for animals should be interpreted with caution.

MIC of different antimicrobials for *M. bovis* isolated from milk were higher (> 128  $\mu\text{g/ml}$ ) compared to MICs for *M. bovis* isolated from lung tissue, indicating resistance according to the Clinical and Laboratory Standards Institute (Soehnlén *et al.*, 2011). In general, *M. bovis* isolated from milk have lower susceptibility to antibiotics, such as spectinomycin, compared to *M. bovis* isolated from BRD cases (Rosenbusch *et al.*, 2005;

Soehnlén *et al.*, 2011). Heuvelink *et al.* (2016) identified relatively high MIC values of *M. bovis* isolates originating from different body sites for commonly recommended antimicrobials against *M. bovis* infections in The Netherlands. Fluoroquinolones inhibited the growth of *M. bovis* most effectively, followed by tulathromycin and oxytetracycline (Heuvelink *et al.*, 2016). However, Lysnyansky and Ayling (2016) reported an increase in antimicrobial resistance of *M. bovis* against tetracyclines, macrolides, lincosamides, aminoglycosides, chloramphenicols, and fluoroquinolones. Further efforts are needed to find the best options to prevent and control *M. bovis* infections to reduce the antimicrobial resistance.

### **2.1.8. Control measures for *Mycoplasma bovis***

To date, the most recommended practice to control *Mycoplasma* infection in dairy herds is to identify the infected animals and to handle those as persistently infected (Gonzalez and Wilson, 2003; Maunsell *et al.*, 2011). While treatment is not recommended, infected cattle should be culled or grouped separately from others, and milking them last in the order is recommended (Pfützner and Sachse, 1996; Gonzalez and Wilson, 2003; Punyapornwithaya *et al.*, 2011). As new animals introduced to the herd is the main risk factor for introducing *Mycoplasma* infection to the herd, farm biosecurity protocols should be up to date, including screening the imported animals and employment of a proper quarantine period (Maunsell *et al.*, 2011). The best way to prevent *M. bovis* infections in a herd is to maintain a closed herd (Maunsell *et al.*, 2011).

As *Mycoplasma* may be detected from bulk milk, that should be tested at least once a month both in naïve dairy herds to monitor the disease-free situation and in herds controlling an endemic disease (Maunsell *et al.*, 2011). Clinical mastitis cases, cows with subclinical mastitis and fresh cows should be sampled to identify *Mycoplasma* IMI (Maunsell *et al.*, 2011; Fox, 2012). To inhibit the transmission of *Mycoplasma* during milking, strict milking hygiene should be maintained (Fox *et al.*, 2005; Bradley *et al.*, 2012). Endemic *Mycoplasma* IMI can be controlled with sampling and strict milking hygiene. However, risk of new *Mycoplasma* IMI outbreaks remains (Fox *et al.*, 2005). Investigation of the whole herd is recommended to identify infected animals and to eliminate *M. bovis* IMI as cows with low SCC may also be infected (Ghadersohi *et al.*, 1999; Maunsell *et al.*, 2011; Pinho *et al.*, 2013).

Despite the aforementioned recommendations, Punyapornwithaya *et al.* (2012) did not identify statistically significant associations between milking hygiene, culling the infected animals and elimination of *Mycoplasma* infection from a dairy herd. *Mycoplasma* IMI was eliminated from the dairy herds participating in the study during a three-months period regardless of the culling intensity of infected animals or maintenance of milking hygiene (Punyapornwithaya *et al.*, 2012).

Contaminated semen, both from breeding bulls and frozen sperm, may predispose the introduction and between-animal transmission of *M. bovis* infection in herds (Gille *et al.*, 2018b; Haapala *et al.*, 2018). Additionally, Gille *et al.* (2018b) identified an absence of calving pen being a risk factor for *M. bovis* antibody and PCR-positive BTM, probably due to increased contact rates between periparturient cows with asymptomatic carriers in the herd. Both aforementioned aspects should be considered when developing control measures for *M. bovis* infections in dairy herds. Furthermore, bedding material contaminated with *Mycoplasma* bacteria should not be used in cattle of any age (Maunsell *et al.*, 2011).

To inhibit the transmission of *M. bovis* infection from dairy cows to calves, pasteurised milk could be fed to calves (Butler *et al.*, 2000). Colostrum should not be pooled, and colostrum contaminated with *M. bovis* should be withheld from calves (Maunsell *et al.*, 2011). Additionally, good housing conditions and management practices to reduce the exposure and maximise host defences decrease the risk of *Mycoplasma* infections among calves (Maunsell *et al.*, 2011). Low stocking density reduces the risk of transmission of *M. bovis* by nose-to-nose contact between calves as well as airborne transmission of the pathogen (Nicholas, 2011). Vaccination against *M. bovis*-associated diseases have been unrewarding (Maunsell *et al.*, 2011). Vaccines have protected cattle from experimentally induced *M. bovis* infection (Chima *et al.*, 1981; Boothby *et al.*, 1987), but in field trials they have been ineffective (Maunsell *et al.*, 2009; Soehlen *et al.*, 2011).

### 3. AIMS OF THE STUDY

The aim of this thesis was to analyze the epidemiology of *M. bovis* intramammary infection in dairy herds. To fulfill this objective, the specific aims covered in this thesis were as follows:

1. Identify the within-herd prevalence and dynamics of *M. bovis* IMI in Estonian dairy herds (I, II).
2. Evaluate the prevalence of *M. bovis* in cow colostrum and clinical mastitis samples (II).
3. Evaluate the associations between *M. bovis* IMI and cow udder health and milk yield and composition (I, III).
4. Compare the genotypes of *M. bovis* strains isolated from cows' clinical mastitis milk samples and calves' respiratory samples (II).
5. Evaluate the elimination of *M. bovis* IMI from infected udder quarters during the dry period (III).

## 4. MATERIALS AND METHODS

### 4.1. Study design

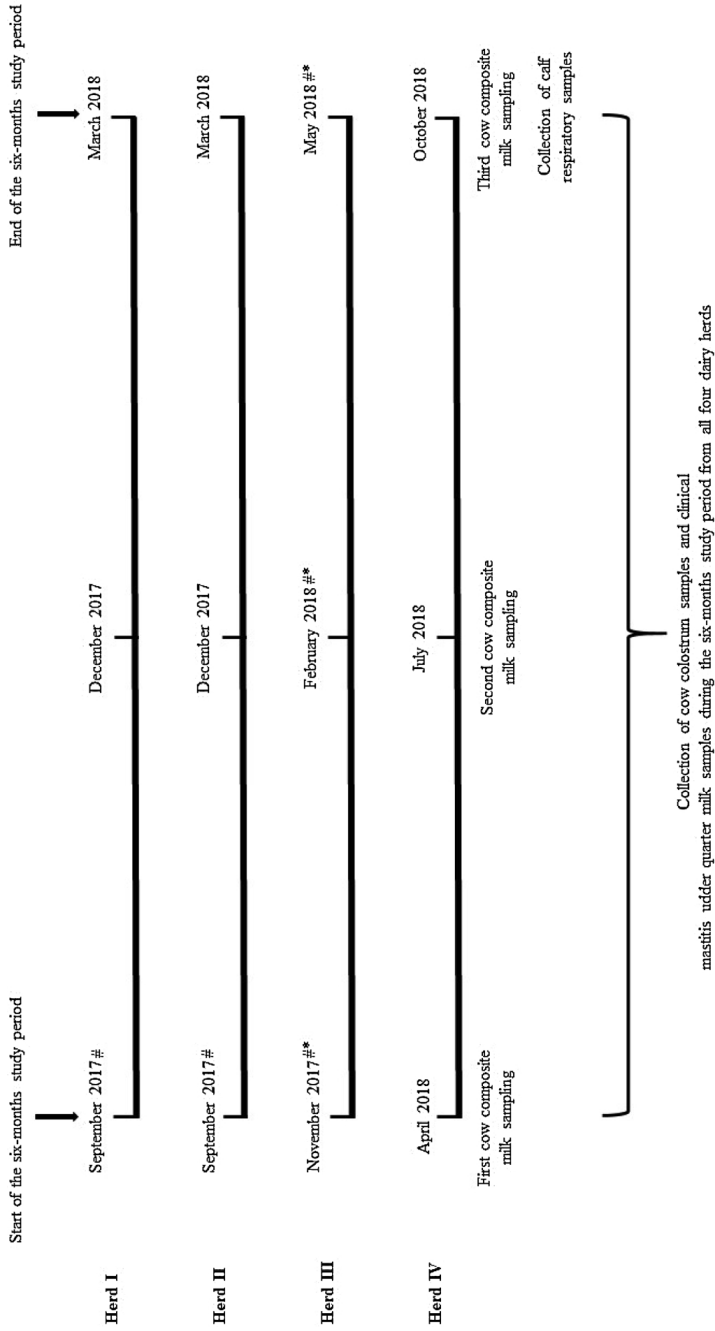
Three studies were designed to cover the objectives of the research. In the three studies, each herd enrolled were positive for *M. bovis* based on herd level bulk tank milk qPCR analysis during the last year (Studies I and III) and during the last six months (Study II) before the beginning of the study.

To evaluate the within-herd prevalence of *M. bovis* IMI in Study I, a cross-sectional study was designed. The study was performed in one Estonian loose-housed dairy herd in November 2014. All lactating dairy cows of the herd were tested.

In Study II, a repeated cross-sectional study design was used to evaluate the dynamics of *M. bovis* IMI in four dairy herds. The study was performed between September 2017 and October 2018, within which a six-month study period was set for each participating herd. All lactating dairy cow CMSs were collected thrice during regular milk-testings in three-month intervals. Additionally, colostrum samples were collected from all calving cows, and udder quarter milk samples were obtained from clinical mastitis cases over the study period. Calf respiratory samples were collected from calves with clinical signs of respiratory disease. Figure 1 illustrates the sampling scheme.

A two-step cross-sectional study was designed in Study III, for which one dairy herd was included. Infectious status of cow udder quarters for *M. bovis* was evaluated at the dry-off and at the first milking after calving for all dairy cows dried off and calved between November 2014 and May 2015.

Herd characteristics are given in Table 2 for Studies I and III as well as in Table 3 for Study II.



**Figure 1.** Sample collection scheme for four study herds between September 2017 and October 2018. A six-month study period was set for each herd. Cow composite milk samples were collected three times from all dairy herds. Cow colostrum samples and clinical mastitis udder quarter milk samples were collected during the whole six-month study period in each herd. Calf respiratory samples were collected once at the last study month of the herd. Time of identification of clinical mastitis udder quarter pools positive for *Mycoplasma bovis* with qPCR is represented as # and time of identifying *Mycoplasma bovis* positive colostrum pools using qPCR as \*

**Table 2.** Key parameters of the herd enrolled in Studies I and III in year 2014

Number of cows	Housing system	Milking system	Milk yield (kg) <sup>1</sup>	Bulk tank SCC <sup>2</sup> (min; max)	Median length of the dry period (days)
611	Free stall	2 x 12 milking parlour	9,916	259, 000; 358, 000	65

<sup>1</sup>Herd average 305-day milk yield<sup>2</sup>Herd average somatic cell count per ml of bulk milk**Table 3.** Key parameters for the four study herds in Study II in year 2018

	Herd 1	Herd 2	Herd 3	Herd 4
First detection of <i>Mycoplasma bovis</i> in bulk tank milk	January 2017	January 2017	November 2014	October 2017
Ct-value of first study month bulk tank milk PCR analysis <sup>1</sup>	34.8	39.4	30.2	33.8
<b>Dairy cows</b>				
Avg. number of cows	591	1,633	552	1,035
Housing system of cows	Free stall	Free stall	Free stall	Free stall
	2 x 12		2 x 12	2 x 12
Milking system	Parallel milking parlour	Carousel	Parallel milking parlour	Parallel milking parlour
Herd avg. 305-day milk yield (kg/cow)	10,502	10,404	9,751	10,095
Herd avg. bulk tank SCC <sup>2</sup>	177,000	158,000	391,000	343,000
Separate grouping of cows with clinical mastitis	Yes	Yes	Yes	Yes
Milking order of clinical mastitis group	Last	Last	Last	Last
Separate grouping of cows with high SCC <sup>3</sup>	No	No	Yes	No
Milking order of high SCC <sup>3</sup> group	Not relevant	Not relevant	Penultimate	Not relevant
<b>Calves ≤6 months of age</b>				
Avg. number of calves	158	422	164	260
Separation from dam	Immediately after birth	Immediately after birth	Immediately after birth	Immediately after birth
Time spent in individual calf box after birth	7 days	7 days	7 days	7 days
Duration of colostrum feeding	2-4 days since birth	2-4 days since birth	4 days since birth	2-4 days since birth

	Herd 1	Herd 2	Herd 3	Herd 4
Feeding after colostrum period	Milk powder	Milk powder	Milk powder (females) Raw milk (bull calves)	Milk powder
Pasteurising colostrum	No	No	No	No

<sup>1</sup> Cycle-threshold value of first study month bulk tank milk polymerase chain reaction analysis

<sup>2</sup> Somatic cell count per ml of milk in bulk tank

<sup>3</sup> Somatic cell count over 200 000 cell per ml of cow composite milk

## 4.2. Sample collection and handling (I-III)

### 4.2.1. Collection of cow composite milk samples (I-II)

For the evaluation of the within-herd prevalence of subclinical *M. bovis* IMI (Study I), 522 cow CMSs were collected during a regular milk-testing in November 2014 from one Estonian dairy herd.

For the evaluation of the changes in within-herd prevalence of *M. bovis* IMI (Study II), cow CMS were collected three times during routine milk recordings in three-month intervals from the study herds (Figure 1).

All CMSs were preserved with bronopol (Broad Spectrum Microtabs II, Advanced Instruments INC., USA) and transported to the milk laboratory of the Estonian Livestock Performance Recording centre in Tartu. After analysis of CMS in Estonian Livestock Performance Recording centre, 1.5 mL of each milk sample was collected and transported to the Estonian University of Life Sciences for storage (Study I, II) and for pooling (Study II).

Cow CMSs (Study I) and pooled cow CMSs (Study II) were stored at  $-18^{\circ}\text{C}$  for further analysis at the Estonian University of Life Sciences.

### 4.2.2. Collection of colostrum samples (II)

Cow composite colostrum samples (40 mL) were collected once at the first milking after calving from all dairy cows calving during the study period from all four study herds. The colostrum samples were frozen and sent to the Estonian University of Life Sciences for pooling and further analysis.

### **4.2.3. Collection of cow clinical mastitis milk samples (II)**

Aseptic udder quarter milk samples were collected from all cows with clinical mastitis. Clinical mastitis was defined as changes in milk appearance alone or together with systemic signs of illness.

Before udder quarter milk sample collection, the teat end was cleaned with 70% ethanol swabs and allowed to dry. After discarding a few streams of milk, samples (2 to 4 mL) were collected into sterile 10-mL plastic tubes. Milk samples were stored at  $-18\text{ }^{\circ}\text{C}$  and transported to the Estonian University of Life Sciences for further analysis.

### **4.2.4. Collection of calf respiratory samples (II)**

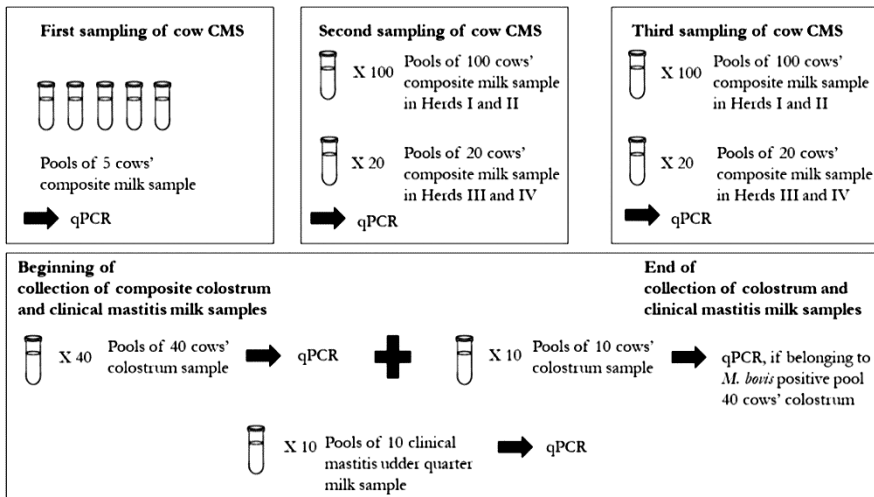
Tracheobronchial lavage (TBL), nasal swab samples or deep pharyngeal swab samples were collected from five to ten calves once from the study herds. Five to ten calves with clinical signs of respiratory disease were chosen for sample collection. Calves having at least one clinical symptom, nasal discharge, coughing, high body temperature ( $\geq 39.3\text{ }^{\circ}\text{C}$ ), difficult breathing or dyspnoe in auscultation, scoring a minimum of 1 according to the calf health scoring chart (Buczinski *et al.*, 2015) were chosen for sampling. For TBL sampling, calf nostrils were cleaned with gauze, and the sampling tube was placed into the upper respiratory tract. Samples were collected with a sterile and flexible sampling catheter (3 mm x 60 cm). Sampling catheter was first introduced to upper respiratory tract through the ventral meatus of the nose. The catheter was introduced further until the caudal pharynx was reached with felt resistance. Further, the catheter was introduced to the trachea during the inspiratory phase of the respiratory cycle (McQuirk and Peek, 2019). After introducing the catheter to the trachea, 40 mL of sterile sodium chloride solution (0.9%) was inserted into the respiratory tract and immediately aspirated. Collected fluid was placed in 10-mL sterile plastic tubes and cooled immediately. Calf nasal swab samples were collected with sterile nasal swabs (Sterile Dry Swab, Copan Diagnostics Inc., USA) inserted 5 to 10 cm into nostrils. For collection of calves' deep pharyngeal swab samples, calf nostrils were cleaned with gauze, and samples were collected with a sterile nasopharyngeal swab (Dryswab<sup>®</sup>, United Kingdom) inserted approximately 25 cm into the upper respiratory tract. Nasal and nasopharyngeal swabs were placed in F-broth for transportation and cooled. After collection of calves' respiratory samples, these were

transported to the Estonian Veterinary and Food Laboratory in Tartu for immediate bacterial cultivation.

#### 4.2.5. Pooling of milk samples (II)

Cow CMSs, colostrum samples and clinical mastitis milk samples were pooled due to expected low prevalence of *M. bovis* in these samples. At the first sample collection, cow CMSs were pooled for five cows' pools. At the second and third sample collection, cow CMSs were pooled for 20 cows' pools in Herds III and IV and for 100 cows' pools in Herds I and II. Clinical mastitis udder quarter milk samples were pooled for ten quarters' pools and colostrum samples for ten cows' and 40 cows' pools, of which ten cows' pools were used for *M. bovis* prevalence estimation. For every pool, 2 ml of individual cow milk sample were used. Pooling and the pooled samples' analysis scheme is illustrated in Figure 2.

All pooled milk samples were stored at  $-18\text{ }^{\circ}\text{C}$  for further analysis at the Estonian University of Life Sciences.



**Figure 2.** Pooling and analytical scheme for cow composite milk, colostrum and clinical mastitis udder quarter milk samples in Study II. Cow colostrum and clinical mastitis milk sample collection was started at the month of first sampling of cow composite milk samples (CMS) and finished at the last month of cow CMS collection. Cow colostrum samples were pooled for 40 and 10 cows' pools. First colostrum pools of 40 cows were analysed with qPCR, following qPCR analysis of 10 cows' colostrum pools belonging to *Mycoplasma (M.) bovis* positive pools of 40 cows. Clinical mastitis udder quarter milk samples were pooled for pools of 10 udder quarter milk samples

#### **4.2.6. Collection of udder quarter milk samples before and after dry period (III)**

Cow aseptic udder quarter milk samples were collected at dry-off and at the first milking after calving from all cows during November 2014 and May 2015. Cow udder quarter milk samples were transported and stored at the Estonian University of Life Sciences at  $-18^{\circ}\text{C}$  for further analysis.

### **4.3. Laboratory analytical methods**

#### **4.3.1. Analysis of cow composite milk samples (I)**

Milk fat (%), protein (%), urea (mg/L), and SCC ( $\times 1,000/\text{mL}$ ) were analysed from CMS with accredited methods using the automatic analyser Combifoss 6000 FC (Hilleroed, Denmark) in the milk laboratory of Estonian Livestock Performance Recording Centre. A calibrated milk meter (Tru-Test Limited, Auckland, New Zealand) was used in the farms during the milk-testing to measure the daily milk yield of each cow. The database of Estonian Livestock Performance Recording Centre was used to collect information about cow parity and DIM of all lactating dairy cows.

#### **4.3.2. Quantitative polymerase chain reaction method (I-III)**

In all studies, a commercial qPCR test kit Mastitis 4B was used to identify the bacterial DNA of *M. bovis* (Studies I-III) in addition to the DNA of *Staph. aureus*, *Strep. agalactiae* and *Strep. uberis* (Study I and III) directly from the CMS, udder quarter milk samples and milk sample pools. Analytical scheme for pooled milk samples in study II is shown in Figure 2.

After thawing, the milk samples were vortexed and from each sample, 500  $\mu\text{L}$  of milk was used for DNA extraction before PCR analysis according to the instructions ([http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction\\_protocol\\_M4B\\_2017.04.26.pdf](http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction_protocol_M4B_2017.04.26.pdf)) from the manufacturer (DNA Diagnostic, Risskov, Denmark). The PCR mixture consisted of 15  $\mu\text{L}$  of the qPCR Master Mix and 5  $\mu\text{L}$  of purified DNA. The real-time PCR instrument thermal cycler Stratagene Mx3005P (Agilent Technologies Inc., Santa Clara, CA) was used for

amplification. The amplification conditions were as follows: 95 °C for 1 min, 1 cycle; 95 °C for 5 s and 60 °C for 25 s, 40 cycles. Cycle threshold (Ct) values were reported for all samples. For all bacteria identified in the analysis, a Ct value of  $\leq 37.0$  was considered a positive result. The assay included controls for the validation of each run including negative DNA extraction controls, internal amplification standard (positive PCR controls), and nontemplate control. The assay was validated on both bacterial strains and milk samples by DNA Diagnostic. According to the internal validation protocol of the laboratory, the sensitivity and specificity of the test kit Mastitis 4B is 100% when tested directly on a bacterial colony.

#### **4.3.3. Microbiological isolation of *Mycoplasma bovis* (II)**

*M. bovis* PCR positive pools of clinical mastitis cases (n = 19) and calves' respiratory samples were cultivated in the Estonian Veterinary and Food Laboratory in Tartu.

Pools of clinical mastitis milk samples were cultivated using an accredited cultivation procedure with a cultivation of an aliquot of 0.1 ml of milk sample in *Mycoplasma*-specific Hayflick agar plates (Pfützner and Sachse, 1996). The aliquot of milk sample was evenly distributed over the plates by sterile spatula and allowed to dry before incubation as described by Olde Riekerink *et al.* (2006). Calves' respiratory samples were cultivated with an isolation method of *Mycoplasma* from tissue material. An aliquot of 0.01 ml of calves' respiratory samples in Friis transport broth were evenly inoculated in Friis agar (Yeary and Nietfeld, 2002; Gabinaitiene *et al.*, 2011). Incubation in a humid atmosphere at 37 °C under 5% CO<sub>2</sub> concentration for 7 days was performed for both Hayflick agar and Friis agar plates. All plates were microscoped daily to detect fried egg-shaped *Mycoplasma* colonies. Suspected colonies were analysed with PCR for *M. bovis* identification (Francoz *et al.*, 2012).

#### **4.3.4. Core genome multilocus sequence typing (II)**

For cg-MLST analysis, *M. bovis* isolates from calves' respiratory samples (n = 5) and clinical mastitis udder quarter milk samples (n = 5) were selected from Herds I and III. DNA isolation and WGS was performed at Finnish Food Authority as described by Haapala *et al.* (2018), with minor modifications. Nextera® Flex DNA Library Preparation kit (Illumina,

USA) was used for library preparation with DNA inputs of 100 ng per library and the libraries were sequenced on a MiSeq instrument (Illumina, USA) using MiSeq Reagent Kit v2 (500-bp) chemistry (Illumina, USA).

#### **4.3.5. Determination of milk haptoglobin (III)**

The concentration of Hp in udder quarter milk samples was determined using the haemoglobin assay described by Makimura and Suzuki (1982), with the modification of tetramethylbenzidine (0.06 mg/ml) as chromogen (Alsemgeest *et al.*, 1994) solution. The method has been adapted to be used for milk, as described by Kalmus *et al.* (2013). Pooled and lyophilised aliquots of bovine acute-phase serum were used to produce standard curves for assay by serial dilution. Optical densities of the formed complex were measured at 450 nm using a spectrophotometer. To calibrate the assay, a bovine sample with a known Hp concentration provided by the European Commission Concerted Action Project (number QLK5-CT-1999-0153) was used. The working range of the assay was 60 to 1,900 mg/L.

The inter- and intra-assay coefficient of variation values for Hp analysis were <12 and <9%, respectively.

#### **4.4. Statistical analysis (I-III)**

A causal diagram was drawn for variables to evaluate their causal associations and identify any confounders before statistical analyses in Studies I and III. The qPCR test results were dichotomised (positive = 1, negative = 0) in all studies for *M. bovis* and for other bacterium in Studies I and III using the qPCR analysis cut-off value ( $\leq 37.0$ ) set by the manufacturer.

In Study I, linear regression models were used to analyse the associations between presence of *M. bovis* in cow CMS and cow milk yield (kg), SCC ( $\times 1,000/\text{mL}$ ), milk protein percentage, fat percentage, and milk urea content (mg/L). Dependent variables in the analysis were cow milk yield, SCC, protein and fat percentage and milk urea content. Natural logarithm transformation was used for SCC, the square root was taken from the milk fat content and an inverse scale was used for milk protein content to achieve the normal distribution of the model residuals. In multivariate models, cow parity (categorised into 1, 2 and  $\geq 3$  lactations)

and DIM (categorised as 1–90, 91–200 and  $\geq 201$  DIM) were inserted to control for confounding effects. In addition to cow status regarding *M. bovis* as the main predictor, presence of *Staph. aureus* and *Strep. agalactiae*, cow parity and DIM were included in the models to control for their confounding effect. Due to low number of qPCR-positive *Strep. uberis* CMS, the cow infection status for that pathogen was not added to the risk factor models. Interaction terms were tested for significance to see whether the combined effect of two mastitis pathogens differs from the individual effects of the pathogens tested (Dohoo *et al.*, 2009). Statistical significance was assumed at  $p \leq 0.05$ . Assumptions of the equal variance of the outcome in all levels of predictor variables and normal distribution of the residuals were checked graphically (Dohoo *et al.*, 2009).

In study II, the within-herd prevalence of *M. bovis* IMI and the prevalence of the pathogen in colostrum and clinical mastitis cases was estimated by analysing pooled cow CMS, colostrum and clinical mastitis samples with qPCR analysis. Separate calculation was done for within-herd prevalence estimation for each herd and sampling round. Pool size varied depending on the sampling time as described above. Prevalence of *M. bovis* in colostrum samples and in clinical mastitis cases was calculated separately for each herd within the six-month study period. Ten cows' colostrum samples were used for prevalence estimation in each herd.

Estimation of the true within-herd prevalence based on pooled samples requires estimations of test sensitivity (Se) and specificity (Sp) (Boelaert *et al.*, 2000). As the objective of whole herd testing is to identify the minimum proportion of truly infected animals, test Se is considered the most important parameter in the calculation of true prevalence based on pooled samples (Murai *et al.*, 2014). In Study II, test Se was set to range uniformly between 77 and 81.3% in all pool sizes, as no perfect Se estimate was available for pooled milk sample analysis with PCR. Justice-Allen *et al.* (2011) described Se of 77% of bulk tank milk PCR analysis for *M. bovis*. This was applied for the lower test Se of 77% (Justice-Allen *et al.*, 2011). Higher limit of Se 81.3% was adapted from a study by Murai *et al.* (2014) evaluating the within-herd prevalence of *M. bovis* IMI based on pooled CMS samples in different pool sizes. This range of test Se should not exaggerate the power of PCR analysis to detect *M. bovis* DNA in low amounts in pooled milk samples. In this study, test Sp was not considered to violate the test results because the objective of a whole herd test is not to gauge truly negative results (non-infected animals)

(Murai *et al.*, 2014). Hence, no positive PCR results were considered false positives in Study II. Test Sp adapted from the manufacturer was set to be 99.95% for all pool sizes.

Calculation of prevalence estimates was performed in statistical programme R (The R Foundation ®) with package prevalence (Centre for Burden and Risk Assessment, 2020) and rjags (Sourceforge, 2020) using model truePrevPools (R Package Documentation, 2020) for estimation of true prevalence from pooled samples (Boelaert *et al.*, 2000; Speybroek *et al.*, 2012). The true prevalence from the apparent prevalence is estimated with a Bayesian framework with credibility intervals (CI) in this model and it considers the relationship between individual and pool test characteristics (Boelaert *et al.*, 2000). In Bayesian estimation, population parameters are assumed to have an intrinsic probability distribution, giving a 95% CI (Gardner, 2002). This indicates that the true values have 95% chance of lying between the limits of CI (Gardner, 2002). As uncertainty on unknown and variable parameters in diagnostic test characteristics is considered in the Bayesian model, a flexible combination of complex equations is possible (Speybroek *et al.*, 2012). Beta posterior distribution, which is the conjugate prior to the proportion/probability parameter in a binomial distribution was used as prior distribution in this study (Dohoo *et al.*, 2014). Calculation of the parameters of beta distribution was made with function betaExpert based on expert information. For this, the best-guess prevalence was specified to vary between 0.1% and 20.0% with the most likely prevalence of 9.0% specified. The calculated alpha and beta estimates of the beta distribution were used in setting the prior beta distribution in the truePrevPools model.

In Study III, the elimination rate of *M. bovis* during the dry period was calculated. Udder quarters were classified as positive (P) or negative (N) for detected *M. bovis* both at dry off and postpartum according to the results of udder quarter milk sample qPCR analysis. Principles of classification of elimination and new IMI rates of *M. bovis* during the dry period were calculated following Dufour and Dohoo (2012) as follows:

$$\text{Elimination} = \frac{(P_{\text{dry}} \text{ and } N_{\text{pp}})}{[(P_{\text{dry}} \text{ and } N_{\text{pp}}) + (P_{\text{dry}} \text{ and } P_{\text{pp}})]}, \text{ and new IMI} = \frac{(N_{\text{dry}} \text{ and } P_{\text{pp}})}{[(N_{\text{dry}} \text{ and } N_{\text{pp}}) + (N_{\text{dry}} \text{ and } P_{\text{pp}})]}.$$

Ndry and Pdry represent infection status at dry-off, and Npp and Ppp represent infection status postpartum.

In Study III, two models were used separately to investigate the associations between milk Hp concentration and *M. bovis* at dry-off and after calving. Presence of udder pathogens *Staph. aureus*, *Strep. agalactiae* and *Strep. uberis* in udder quarter milk samples at dry-off and post-partum was added to models as confounders. Cow parity was categorised into 1, 2, 3 and  $\geq 4$  lactations and DIM into  $< 300$  and  $\geq 301$ . The presence of blood in milk samples was evaluated visually and dichotomised (present = 1, absent = 0). To achieve a normal distribution of the outcome variable, the inverse square root (1/square root) transformation for Hp concentration was used. According to the causal diagram, the presence of blood in the milk sample, cow parity, DIM and the length of the dry period were possible confounders in both models. A mixed tobit regression model was used for estimating the associations between the milk Hp concentration and the presence of udder pathogens in udder quarter milk samples at dry-off. The model was chosen due to 17.8% of the milk samples being under the detection limit for Hp concentrations at dry-off, which would violate the regression model's assumptions. In the tobit regression, all cases above (or below) a specific threshold value were censored, although these cases remained in the analysis. Hence, all cases of Hp concentrations under detection limit were censored, although these Hp concentrations remained in the analysis. A mixed-effect linear regression model was used to estimate the associations between milk Hp level and the presence of tested udder pathogens in udder quarter milk samples after calving. The cow was included as a random factor to both mixed tobit regression and mixed-effect linear regression models. In both models, interaction terms were tested for significance to determine whether the combined effect of two udder pathogens differed from the sum of the individual effects of the pathogens tested. Assumptions of the equal variance of the outcome in all levels of predictor variables and normal distribution of the residuals were checked graphically (Dohoo *et al.*, 2009).

Stata IC 10 (StataCorp, College Station, TX) software was used for statistical analyses in Studies I and III.

## 5. RESULTS

### 5.1. The prevalence of *Mycoplasma bovis* intramammary infection

#### 5.1.1. Within-herd prevalence of *Mycoplasma bovis* intramammary infection (I and II)

In Study I, 522 cow CMSs were collected. *M. bovis* alone was detected in 15.0% (n = 30) of pathogen-positive milk samples and in combination with *Strep. agalactiae* in 19.5% (n = 39) of pathogen-positive milk samples.

In study I, the within-herd prevalence of *M. bovis* IMI was 17.2 % (n = 90; 95% CI 14.1-20.8) in November 2014.

In total, 1,533 cow CMSs were collected from Herd I, 4,549 CMSs from Herd II, 1,427 CMSs from Herd III and 2,835 CMSs from herd IV during the Study II.

The estimated within-herd prevalence of *M. bovis* IMI in four study herds during the Study II is shown in Table 4. Over four farms, the estimated within-herd prevalence of *M. bovis* IMI ranged between 0.4% (95% CI 0.0; 0.7) and 12.3% (95% CI 9.7; 15.2) during the study period.

**Table 4.** Within-herd prevalence of *Mycoplasma bovis* intramammary infection in study herds during a six-month study period evaluated by pooled cow composite milk samples

Sampling	First sampling			Second sampling			Third sampling		
	Herd (avg. number of cows in herd)	n positive / n total <sup>1</sup>	% <sup>2</sup>	95% CI	n positive / n total <sup>1</sup>	% <sup>2</sup>	95% CI	n positive / n total <sup>1</sup>	% <sup>2</sup>
Herd I (n = 591)	5 / 96	4.7	2.9; 6.8	0 / 5 <sup>3</sup>	1.0	0.1; 1.7	0 / 6 <sup>3</sup>	0.8	0.1; 1.4
Herd II (n = 1,633)	9 / 276	3.4	2.3; 4.6	0 / 15 <sup>3</sup>	0.4	0.0; 0.7	0 / 15 <sup>3</sup>	0.4	0.0; 0.7
Herd III (n = 552)	28 / 92	12.3	9.7; 15.2	10 / 25	4.6	3.0; 6.4	5 / 23	3.2	1.9; 4.8
Herd IV (n = 1,035)	31 / 187	7.8	6.2; 9.5	10 / 47	2.8	1.9; 3.8	22 / 47	4.9	3.6; 6.4
Pool size	5 cows			20 / 100 <sup>3</sup> cows			20 / 100 <sup>3</sup> cows		

<sup>1</sup>Number of *Mycoplasma bovis* positive pools / total number of pools analysed

<sup>2</sup>Estimated within-herd prevalence of *Mycoplasma bovis*

<sup>3</sup>Pools of hundred cows

### 5.1.2. The prevalence of *Mycoplasma bovis* in cow colostrum and clinical mastitis samples (II)

The prevalence of *M. bovis* in cow colostrum samples and in clinical mastitis udder quarter milk samples was calculated over four farms. During the study, 1,264 colostrum samples were collected from four study herds. Ten *M. bovis* positive colostrum pools (n = 28 tested) of ten cows' was identified in Herd III. *M. bovis* was not detected from pools of ten cows' colostrum in Herd I (n = 60 tested), Herd II (n = 23 tested) or Herd IV (n = 16 tested). In total, 707 udder quarter milk samples were collected from clinical mastitis cases from the four study herds. Six *M. bovis*-positive pools of clinical mastitis cases originated from Herd I (n = 26 tested), one *M. bovis*-positive pool of originated from Herd II (n = 20 tested), and 13 *M. bovis*-positive pools of clinical mastitis cases originated from Herd III (n = 23 tested). From Herd IV, seven pools of clinical mastitis cases were available, but none of these were *M. bovis*-positive in PCR analysis.

The estimated prevalence of *M. bovis* in cow colostrum in the study herds ranged between 1.7% (95% CI 0.2; 2.8) and 4.7% (95% CI 2.7; 7.1) evaluated by pools of ten cows' colostrum samples (Table 5). In clinical mastitis cases, the estimated prevalence of *M. bovis* in the study herds during the study ranged between 3.7% (1.7; 6.4) and 11.0% (7.5; 15.2) (Table 6).

**Table 5.** Prevalence of *Mycoplasma bovis* in cow colostrum in study herds during a six-month study period evaluated by pooled colostrum samples

Herd	<i>Mycoplasma bovis</i> positive pools	Total number of pools analysed	% <sup>1</sup>	95% CI
Herd I	0	23	2.8	0.2; 4.6
Herd II	0	60	1.7	0.2; 2.8
Herd III	4	28	4.7	2.7; 7.1
Herd IV	0	16	3.4	0.3; 5.6

<sup>1</sup> Estimated prevalence of *Mycoplasma bovis* in colostrum calculated by pools of ten colostrum samples

**Table 6.** Prevalence of *Mycoplasma bovis* in clinical mastitis cases in study herds during a six-month study period evaluated by pooled udder quarter milk samples

Herd	<i>Mycoplasma bovis</i> positive pools	Total number of pools analysed	% <sup>1</sup>	95% CI
Herd I	6	26	5.9	3.7; 8.7
Herd II	1	20	3.7	1.7; 6.4
Herd III	13	23	11.0	7.5; 15.2
Herd IV	0	7	5.0	0.3; 8.5

<sup>1</sup> Estimated prevalence of *Mycoplasma bovis* in clinical mastitis cases calculated by pools of ten udder quarter milk samples

## 5.2. Associations between the *Mycoplasma bovis* IMI and cow production indices (I)

A lower (on average  $-3.0$  kg) daily milk yield was associated with the presence of *M. bovis* in cow CMS compared to cows negative to *M. bovis*. The SCC was significantly higher in cow CMS positive for *M. bovis* compared to cows negative for *M. bovis*. In the CMS of cows positive for *M. bovis*, the milk fat and urea content were lower compared to *M. bovis*-negative CMS. No significant association was identified between cow *M. bovis* IMI status and milk protein content. The results of multivariate linear regression models evaluating the associations between the udder pathogens and cow production indices are presented in Tables 7–11.

**Table 7.** Results of multivariable linear regression model of association between detected mastitis pathogens in cow composite milk samples and daily milk yield (kg) of dairy cows (n=520)

	Coefficient	95 % CI	p-value	Wald test
<i>M. bovis</i> <sup>1</sup> neg (n = 430)	0			
<i>M. bovis</i> pos (n = 90)	-3.0	-5.2; -0.8	0.007	
<i>Staph. aureus</i> <sup>2</sup> neg (n = 467)	0			
<i>Staph. aureus</i> pos (n = 53)	0.8	-1.8; 3.5	0.546	
<i>Strep. agalactiae</i> <sup>3</sup> neg (n = 372)	0			
<i>Strep. agalactiae</i> pos (n = 148)	-4.0	-5.9; -2.2	<0.001	
1. lactation (n = 220)	0			<0.001
2. lactation (n = 159)	3.7	1.9; 5.5	<0.001	
≥3. lactation (n = 141)	3.3	1.4; 5.3	<0.001	
<90 DIM (n = 133)	0			<0.001
91–200 DIM (n = 170)	-4.4	-6.5; -2.3	<0.001	
≥201 DIM (n = 217)	-13.6	-15.6; -11.6	<0.001	
Intercept	36.0	34.2; 37.9	<0.001	

<sup>1</sup> *Mycoplasma bovis*

<sup>2</sup> *Staphylococcus aureus*

<sup>3</sup> *Streptococcus agalactiae*

**Table 8.** Results of multivariable linear regression model of association between somatic cell count in cow composite milk samples and mastitis pathogens (n=520)

	Coefficient <sup>4</sup>	95 % CI	p-value	Wald test
<i>M. bovis</i> <sup>1</sup> neg (n = 430)	0			
<i>M. bovis</i> pos (n = 90)	0.8	0.5; 1.1	<0.001	
<i>Staph. aureus</i> <sup>2</sup> neg (n = 467)	0			
<i>Staph. aureus</i> pos (n =53)	0.9	0.5; 1.3	<0.001	
<i>Strep. agalactiae</i> <sup>3</sup> neg (n =372)	0			
<i>Strep. agalactiae</i> pos (n =148)	0.6	0.3; 0.8	<0.001	
1. lactation (n = 220)	0			<0.001
2. lactation (n = 159)	0.1	-0.2; 0.4	0.459	
≥3. lactation (n = 141)	0.6	0.3; 0.8	<0.001	
<90 DIM (n =133)	0	-0.2; 0.4		<0.001
91–200 DIM (n = 170)	0.1	0.2; 0.8	0.399	
≥201 DIM (n =217)	0.5		<0.001	
Intercept	3.7	3.4; 4.0	<0.001	

<sup>1</sup> *Mycoplasma bovis*

<sup>2</sup> *Staphylococcus aureus*

<sup>3</sup> *Streptococcus agalactiae*

<sup>4</sup> estimates are in logarithmic scale

**Table 9.** Results of multivariable linear regression model of association between milk fat in cow composite milk samples and mastitis pathogens (n=520)

	Coefficient <sup>4</sup>	95 % CI	p-value	Wald test
<i>M. bovis</i> <sup>1</sup> neg (n = 430)	0			
<i>M. bovis</i> pos (n = 90)	-0.05	-0.1; -0.004	0.035	
<i>Staph. aureus</i> <sup>2</sup> neg (n = 467)	0			
<i>Staph. aureus</i> pos (n =53)	-0.1	-0.07; 0.05	0.732	
<i>Strep. agalactiae</i> <sup>3</sup> neg (n =372)	0			
<i>Strep. agalactiae</i> pos (n =148)	0.05	0.002; 0.09	0.041	
1. lactation (n = 220)	0			0.021
2. lactation (n = 159)	-0.06	-0.1; -0.01	0.011	
≥3. lactation (n = 141)	-0.05	-0.1; -0.001	0.046	
<90 DIM (n =133)	0			<0.001
91–200 DIM (n = 170)	-0.03	-0.08; 0.02	0.233	
≥201 DIM (n =217)	0.08	0.04; 0.1	0.001	
Intercept	2.0	1.95; 2.04	<0.001	

<sup>1</sup> *Mycoplasma bovis*

<sup>2</sup> *Staphylococcus aureus*

<sup>3</sup> *Streptococcus agalactiae*

<sup>4</sup> estimates are on a square root scale

**Table 10.** Results of multivariable linear regression model of association between milk protein in cow composite milk samples and mastitis pathogens (n=520)

	Coefficient <sup>4</sup>	95 %CI	p-value	Wald test
<i>M. bovis</i> <sup>1</sup> neg (n = 430)	0			
<i>M. bovis</i> pos (n = 90)	0.001	-0.005; 0.007	0.706	
<i>Staph. aureus</i> <sup>2</sup> neg (n = 467)	0			
<i>Staph. aureus</i> pos (n = 53)	0.002	-0.006; 0.009	0.674	
<i>Strep. agalactiae</i> <sup>3</sup> neg (n = 372)	0			
<i>Strep. agalactiae</i> pos (n = 148)	-0.006	-0.01; -0.0004	0.034	
1. lactation (n = 220)	0			0.282
2. lactation (n = 159)	0.003	-0.002; 0.008	0.214	
≥3. lactation (n = 141)	0.004	-0.002; 0.009	0.190	
<90 DIM (n = 133)	0			<0.001
91–200 DIM (n = 170)	-0.02	-0.02; -0.01	<0.001	
≥201 DIM (n = 217)	-0.04	-0.05; -0.04	<0.001	
Intercept	0.3	0.30; 0.31	<0.001	

<sup>1</sup> *Mycoplasma bovis*

<sup>2</sup> *Staphylococcus aureus*

<sup>3</sup> *Streptococcus agalactiae*

<sup>4</sup> estimates are on inverse scale (negative estimate means higher content of protein)

**Table 11.** Results of multivariable linear regression model of association between milk urea (mg/L) in cow composite milk samples and mastitis pathogens (n=520)

	Coefficient	95 % CI	p-value	Wald test
<i>M. bovis</i> <sup>1</sup> neg (n = 430)	0			
<i>M. bovis</i> pos (n = 90)	-15.6	-25.6; -5.6	0.002	
<i>Staph. aureus</i> <sup>2</sup> neg (n = 467)	0			
<i>Staph. aureus</i> pos (n = 53)	-6.0	-18.2; 6.3	0.339	
<i>Strep. agalactiae</i> <sup>3</sup> neg (n = 372)	0			
<i>Strep. agalactiae</i> pos (n = 148)	-7.8	-16.4; 0.7	0.072	
1. lactation (n = 220)	0			<0.001
2. lactation (n = 159)	-3.8	-12.2; 4.7	0.379	
≥3. lactation (n = 141)	-16.8	-25.7; -7.9	<0.001	
<90 DIM (n = 133)	0			<0.001
91–200 DIM (n = 170)	28.2	18.7; 37.7	<0.001	
≥201 DIM (n = 217)	17.1	8.1; 26.2	<0.001	
Intercept	159.4	150.8; 168.0	<0.001	

<sup>1</sup> *Mycoplasma bovis*

<sup>2</sup> *Staphylococcus aureus*

<sup>3</sup> *Streptococcus agalactiae*

### 5.3. Associations between the milk haptoglobin concentration and the presence of *Mycoplasma bovis* in cow udder quarter milk samples (III)

Associations between the presence of *M. bovis* in udder quarter milk samples at dry off and post-partum and milk haptoglobin concentration were evaluated in Study III. The presence of *M. bovis* in udder quarter milk sample was not significantly associated with milk Hp concentration either at dry-off or at the first milking after calving.

### 5.4. Comparison of genomes of *Mycoplasma bovis* isolates from calf respiratory and cow clinical mastitis samples (II)

In total, 33 respiratory tract samples were collected from calves from four study herds. Five *M. bovis* isolates were identified from the respiratory samples. *M. bovis* isolates were identified from eight udder quarter milk sample pools from clinical mastitis cases (Table 12).

**Table 12.** *Mycoplasma bovis* isolates from four dairy herds identified by cultivation on Hayflick agar at 37 °C and 10% CO<sub>2</sub> and used for core genome multilocus sequence typing

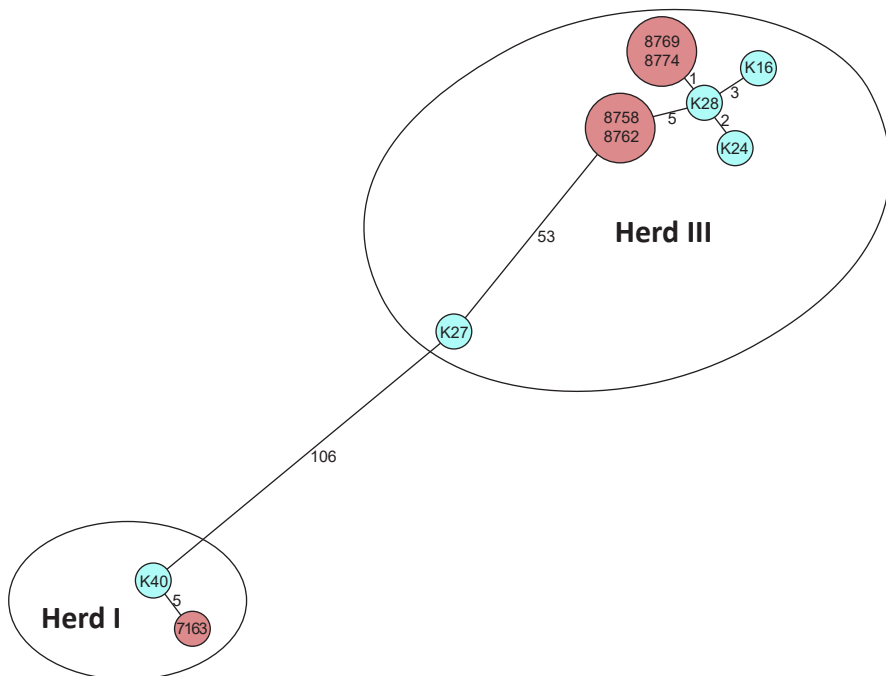
Herd	Number of samples taken	Sample type	Number of isolates
I	2	TBL <sup>1</sup>	1
	6	Clinical mastitis case <sup>2</sup>	1
II	6	Nasal swab	0
	2	Deep nasopharyngeal swab	0
	1	Clinical mastitis case <sup>2</sup>	0
III	8	Nasal swab	4
	13	Clinical mastitis case <sup>2</sup>	4
IV	15	Deep nasopharyngeal swab	0
	5	Clinical mastitis case <sup>2</sup>	0

<sup>1</sup>Tracheobronchial lavage

<sup>2</sup> Udder quarter milk sample pool of 10 udder quarters deriving from clinical mastitis cases

One calf respiratory isolate (7163) and cow clinical mastitis (K40) isolate originated from Herd I, whereas four calf respiratory isolates (8769, 8774, 8758, 8762) and four cow clinical mastitis isolates (K27, K28, K16, K24) originated from Herd III (Figure 3) from isolates selected for cg-MLST analysis.

The cg-MLST schema targets covered 58.2% of the reference genome. In total, 527 cg-MLST allele-called targets were extracted and compared with each other from the 10 isolates in cg-MLST analysis. *M. bovis* isolates from calves' respiratory samples and cows' milk samples clustered together within herds. The cow mastitis and calves' respiratory strains had allele differences of 5 in Herd I. In Herd III, mastitis and respiratory strains had allele differences 1 to 5, with an exception in sample K27, which had an allele difference of 53 compared to other strains from the herd. The allele difference between Herd I and Herd III was at least 106 (Figure 3).



**Figure 3.** Minimum spanning tree constructed from the core genome multilocus sequence typing allele profiles from *Mycoplasma bovis* strains isolated from cow clinical mastitis cases (turquoise circles) and calves' respiratory samples (red circles) from two dairy herds (Herds I and III). Sample ID-numbers are given inside the red and turquoise circles. Allele differences between isolates are shown in red

### 5.5. Elimination of *Mycoplasma bovis* during the dry period (III)

In Study III, 1,001 udder quarter milk samples were collected at dry-off (n = 510) and after calving (n = 491) from 133 dairy cows. At dry-off, 191 (37.5%) udder quarter samples out of 510 tested samples were positive for one or more of the detected udder pathogens. *M. bovis* was presented in 2.9% (n = 15) of the udder quarter milk samples at dry-off. After calving, 57 (11.6%) of 491 udder quarter milk samples were positive for detected udder pathogens. *M. bovis* was identified in 17 (3.5%) udder quarter milk samples.

For *M. bovis*, the elimination rate during the dry period was 86.7% (n = 13 *M. bovis*-negative udder quarter milk samples post-calving, positive at dry-off / n = 15 *M. bovis*-positive udder quarter milk samples at dry-off) and a new IMI rate of 3.0% (n = 15 *M. bovis*-positive udder quarter milk samples post-calving, negative at dry-off / n = 498 *M. bovis* negative udder quarter milk samples at dry-off).

## 6. DISCUSSION

### 6.1. The within-herd prevalence of *Mycoplasma bovis* intramammary infection (I and II)

In a cross-sectional study in November 2014, we identified an *M. bovis* IMI within-herd prevalence of 17.2% in one dairy herd (Study I). In the Study II, the within-herd prevalence of *M. bovis* IMI varied between four dairy herds during a six-month study period. In general, the within-herd prevalence of *M. bovis* IMI was low, ranging between 0.4% and 12.3%.

The within-herd prevalence of *M. bovis* udder infection has not been widely studied in European dairy herds. Most of the previous studies focused on clinical mastitis cases or on herd prevalence of *M. bovis* (Filioussis *et al.*, 2007; Arcangioli *et al.*, 2011; Radaelli *et al.*, 2011; Passchyn *et al.*, 2012). Previously, Brown *et al.* (1990) identified a 5.7% (n = 1,535) within-herd prevalence of *Mycoplasma* in one herd. According to the study of Brown *et al.* (1990) a three times higher within-herd prevalence of *M. bovis* IMI was found in Study I.

The study herd of Study I was also included in Study II (Herd III). The within-herd prevalence of 17.2% identified in Study I decreased to 3.2% at the lowest in Study II during a three-year period between the two studies. Epidemiologic phase of the infection may affect the within-herd prevalence of IMI. Additionally, the within-herd prevalence of *M. bovis* IMI may vary with time, and probably, the longer the infection circulates in the farm, the lower the within-herd prevalence might be due to better immunity of the cows for *M. bovis* IMI. Hence, in herds positive to *M. bovis* at all three samplings in Study II, circulation of the pathogen occurs in dairy cows, but assumptions whether this is due to persistent IMI or cure and re-occurrence of IMI cannot be made. The study herds were selected based on an *M. bovis*-positive bulk tank milk sample without knowledge about the epidemiologic phase of the *M. bovis* IMI. The within-herd prevalence was close to zero after the first CMS sampling in study Herds I and II, whereas in the herds III and IV, *M. bovis*-positive cow CMS pools were identified during the whole study period. Biddle *et al.* (2003) described an intermittent shedding of *Mycoplasma* organism in cows with *Mycoplasma* IMI. Additionally, secreting low concentrations of *Mycoplasma* organisms (106 cells/ml) in CMS is described (Biddle *et al.*,

2003). These aspects may affect the within-herd prevalence of *M. bovis* in the four study herds.

## **6.2. The prevalence of *Mycoplasma bovis* in cow colostrum and clinical mastitis samples (II)**

The estimated prevalence of *M. bovis* in cow colostrum samples was low over all four dairy herds in Study II, indicating that in endemically infected dairy herds, the shedding of *M. bovis* in colostrum is minimal. The transmission of *M. bovis* infections from dairy cows to calves is commonly thought to occur via feeding unpasteurised milk to calves (Maunsell and Donovan, 2009). As none of the study herds pasteurised colostrum, the transmission of *M. bovis* to calves through colostrum could be possible.

*M. bovis* may cause both subclinical and clinical mastitis (Bushnell, 1984; Pothmann *et al.*, 2015; Ruegg and Erskine, 2015). The estimated prevalence of *M. bovis* in clinical mastitis cases ranged between 3.7% and 11.0% in the herds during the study. Similar results were described in a study by Vähänikkilä *et al.* (2019), who collected 3,268 udder quarter milk samples from clinical and subclinical mastitis cases and identified only 51 cows with *M. bovis* clinical mastitis in recently infected herds during a two-year study period.

## **6.3. Estimation of intramammary *Mycoplasma bovis* within-herd prevalence from cow composite and pooled milk samples with qPCR (I and II)**

Cow CMSs are usually used to detect subclinical mastitis and IMI, when it is difficult to identify the infected udder quarter. However, milk originating from non-infected udder quarters may lower the sensitivity in IMI pathogen detection, due to a dilution effect (Reyher and Dohoo, 2011). In Study I and II, we used commercial qPCR analysis to detect *M. bovis* from cow CMS. A high sensitivity of the PCR method to detect udder pathogens causing IMI is reported, also when cow CMS are used (Friendship *et al.*, 2010; Koskinen *et al.*, 2010; Murai *et al.*, 2014; Nyman *et al.*, 2016).

A carry-over of the udder pathogen DNA may occur when CMSs are collected with automated milk metres (Study I and II). Milk from a

previously milked cow is mixed with milk from the cow currently being milked, leading to false-positive PCR test results (Løvendahl *et al.*, 2010; Mahmmod, 2015). In conventional milking systems, the probability of carry-over ranges from 2 to 3.5% (Løvendahl and Bjerring, 2006; Løvendahl *et al.*, 2010). The carry-over effect may be associated with the Ct-value cut-off (Mahmmod *et al.*, 2014). Lower cut-off values in discriminating positive and negative test results lower the sensitivity of the test but reduce the number of false-positive test results that may occur due to carry over (Mahmmod *et al.*, 2014). Cut-off value was set to be  $\leq 37.0$  in Study I, which should be low enough to rule out most of the false-positive results and hence decrease the probability that contaminated samples are classified as positive (Mahmmod *et al.*, 2014). In Study II, cow CMS samples were pooled, and the dilution effect of pooled CMS decreased the probability of false-positive qPCR test results due to carry-over effect.

We estimated the within-herd prevalence of *M. bovis* using pooled cow CMSs in Study II. Previous studies have used both pooled udder quarter milk samples (Soltau *et al.*, 2017) and CMS (Murai *et al.*, 2014) in IMI within-herd prevalence estimation. If assumed individual-animal prevalence is  $<10\%$ , pooled testing is justified (Cowling *et al.*, 1999). However, comparing to individual animal testing, pooling causes a potential decrease in sensitivity (Christensen and Gardner, 2000). Additionally, cows do not excrete a steady number of bacteria with milk all the time (Nyman *et al.*, 2016). We used pools of five, 20 and 100 cows in within-herd prevalence estimation and 40 and ten cows' pools estimating the prevalence in cow colostrum. Especially in the pools of more than 20 cows, the bacterial DNA might have been below the detection limit of qPCR analysis. However, using pooled cow CMS, Murai *et al.* (2014) identified a Se of 81.3% for *M. bovis* IMI within-herd prevalence estimation strategy with analysing first hundred cows' CMS pools with qPCR following qPCR analysis of 50 cows' CMS pools and then individual cow CMS analysis by culture. In Study II, the Se for the calculation of prevalence estimations was set to a range between 77 and 81.3%. Additionally, prevalence estimates were calculated based on a Bayesian framework, which is an exact method and allows the parameters to be unknown (Cowling *et al.*, 1999). With these methods, the prevalence estimates were adjusted to imperfect Se when using pooled milk samples and the power of PCR analysis to detect low amounts of *M. bovis* DNA from large pools is not overestimated.

#### **6.4. The associations between cow *Mycoplasma bovis* intramammary infection and milk yield and milk composition (I)**

On average, cow daily milk yield was 3.0 kg lower in cows positive for *M. bovis* IMI, evaluated by cow CMS qPCR analysis compared to cows negative for *M. bovis*. Hence, the economic impact of *M. bovis* IMI on dairy farm production is most probably negative. On the contrary Al-Farha *et al.* (2017) did not find a significant decrease in daily milk yield in cows positive for *M. bovis*. However, *Mycoplasma* co-infection with other udder pathogens resulted in lower daily milk yield. However, considering the negative economic impact of mastitis to dairy producers, *M. bovis* mastitis should also be effectively controlled in dairy operations.

In this study, negative association was found between *M. bovis* IMI and some of the milk composition parameters. In *M. bovis*-positive cows the milk fat and urea content were lower than in *M. bovis*-negative cows. Changes in milk fat composition and decrease in milk fat content may occur due to spontaneous lipolysis during the IMI (Forsbäck *et al.*, 2010). Urea is formed in the liver from ammonium and absorbed to the milk from the blood in a stable form (Hayton *et al.*, 2012). However, many factors influence the milk urea content, such as altered dry matter intake, or the lack of rumen degradable protein and energy in feed ratio (Rezamand *et al.*, 2007; Hayton *et al.*, 2012). Therefore, strong conclusions cannot be made about the causality of *M. bovis* IMI and milk urea content. IMI usually causes a decrease in milk protein levels (Rezamand *et al.*, 2007). However, we did not find a significant association between the presence of *M. bovis* in milk samples and milk protein content. Further studies using a larger sample size are needed to confirm the associations between *M. bovis* IMI and milk yield as well as milk composition parameters to estimate the economic impact of *M. bovis* IMI and cow well-being on the herd level.

#### **6.5. Inflammatory response in the *Mycoplasma bovis* infected udder quarters before and after dry period (III)**

We did not find a significant association between the milk Hp concentration and the presence of *M. bovis* in udder quarter milk samples at dry off or at the first milking after calving. To our knowledge, no studies have evaluated the presence of *M. bovis* in udder quarters and local inflammatory response in mammary glands measured via milk Hp

concentration. Therefore, further studies should evaluate the factors affecting milk Hp concentration in *M. bovis*-positive udder quarters.

## 6.6. Comparison of *Mycoplasma bovis* strains (II)

We identified that *M. bovis* strains originating from calves' respiratory tract and cow clinical mastitis cases clustered together within herds. However, the allele difference between *M. bovis* strains from two herds was large, indicating circulation of different strains in the two herds. Calves' respiratory and cows' clinical mastitis *M. bovis* strains had low allele differences within herds. In Herd III, one exception was a clinical mastitis *M. bovis* strain, which differed from other pathogen strains originating from calves' respiratory samples and clinical mastitis udder quarter milk samples. Subclinically infected and imported animals may transfer new *M. bovis* strains to a naïve dairy herd (Fox, 2012). In 2016, Herd III created a beef unit housed close to a dairy youngstock barn and direct contact was possible between beef cattle and dairy heifers over the fence on pasture. This may have caused the transmission of new *M. bovis* strains to the dairy unit.

Herd-specific *M. bovis* strains and a close relationship between strains originating from animals in different ages refers to transmission of pathogen between dairy cows and calves. Contaminated milk is commonly thought to be a transmitter of *M. bovis* between dairy cows during milking and between cows and calves (Maunsell and Donovan, 2009; Fox 2012). Calves were fed with individual cow colostrum originating from cows from the same study herd followed by milk from post-partum cows for two to four days after birth and then milk replacer until weaning in all the study herds. Additionally, calves were separated from their dams immediately after birth, but housed in individual boxes in a calving barn during the first week of life. As *M. bovis* may survive in cool and humid conditions for long periods (Pfützner, 1984) and air-borne transmission of *M. bovis* is also described (Nicholas, 2011), a transmission of *M. bovis* between dairy cows and calves is possible during the first week of calves' lives. After moving to group pens, direct transmission between calves and indirect transmission of *M. bovis* through farm personnel's contaminated hands and equipment is possible. Further molecular epidemiologic and longitudinal studies should evaluate the specific transmission routes of *M. bovis* between adult dairy cows and calves.

### 6.7. Elimination of *Mycoplasma bovis* during the dry period (III)

A high elimination rate for *M. bovis* during the dry period was identified in Study III. *M. bovis* is described to be resistant to many antibiotics, including cloxacillin (Rosenbusch *et al.*, 2005). In Study III, all cows were treated with cloxacillin-based dry cow antibiotic product (Noroclox DC, 600 mg, Norbrook Laboratories Limited, Newry, Ireland) at dry-off. Usually, *M. bovis* IMI is considered untreatable with dry cow therapy (Ruegg and Erskine, 2015). As dry cow antibiotic therapy should be ineffective against *M. bovis* IMI, the elimination of *M. bovis* during the dry period is probably caused by other factors. These factors may include altered cow immunity or unsuitable conditions for *Mycoplasma* cells in dry mammary glands. However, the sample size in this study was small, and therefore the results are only indicative. Future studies should evaluate the factors affecting the elimination of *M. bovis* during the dry period in a larger study population.

Udder pathogens can be detected from milk with high sensitivity using PCR (Koskinen *et al.*, 2010). In Study III, we collected cow udder quarter milk samples only once before and after the dry period. Cows do not excrete a steady number of bacteria with milk all the time (Nyman *et al.*, 2016), and in this study, the amount of bacterial DNA in negative udder quarter milk samples may have been below the qPCR detection limit, giving false-negative results. Similarly, qPCR also detects the bacterial DNA from nonviable bacteria (Nyman *et al.*, 2016; Parker *et al.*, 2018). Therefore, qPCR positive udder quarter milk samples in Study III may have been falsely classified as positive due to bacterial DNA from bacteria already killed by the cows' immune system. By using repeated udder quarter milk sampling, the probability of false-negative and false-positive qPCR test results could have been decreased.

## 7. CONCLUSIONS

*M. bovis* IMI was negatively associated with cow daily milk yield, milk composition and udder health. Herds should be tested for the presence of *M. bovis* infection and effective control measures should be used in infected herds to reduce economic losses.

The within-herd prevalence of *M. bovis* IMI was 17.2% by testing CMSs of 522 lactating dairy cows using qPCR in one dairy herd in November 2014. The within-herd prevalence of *M. bovis* IMI varied in four dairy herds during a six-month study period between years 2017–2018. In general, the within-herd prevalence of *M. bovis* was low. Shedding of *M. bovis* may depend on the phase of the epidemic and therefore, repeated testing of animals is recommended to assess the extent of the infection.

In endemically infected dairy herds, the prevalence of *M. bovis* in cow colostrum is low, indicating a minimal risk of transmission of *M. bovis* to calves via colostrum. Herd-specific *M. bovis* strains isolated from calves and cows indicate a within-herd circulation of the pathogen between different age groups in endemically infected dairy herds. Further studies should evaluate the importance of different within-herd transmission routes of *M. bovis* infection.

The incidence of *M. bovis*-induced clinical mastitis is low in endemically infected dairy herds. Most probably, *M. bovis* is not the major pathogen causing clinical mastitis in herds with low pathogen within-herd prevalence.

Most of the udder quarter milk samples positive to *M. bovis* at dry off (86.7%) turned to pathogen-negative during the dry period. Further studies should evaluate the elimination of *M. bovis* IMI during the dry period with larger sample size. No significant association between the milk Hp concentration and the presence of *M. bovis* in udder quarter milk samples at dry off or at the first milking after calving was identified. Associations between *M. bovis* IMI and local inflammatory response in the udder should be studied further.

Further studies should also evaluate the dynamics of *M. bovis* IMI during a longer period of time to understand better the infection patterns over

time. Additionally, the effect of udder health programs for controlling the within-prevalence of *M. bovis* IMI in dairy herds should be studied. The importance of different within-herd transmission routes of *M. bovis* is also necessary.

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## 9. SUMMARY IN ESTONIAN

### *Mycoplasma bovis*'e põhjustatud udaranakkuse epidemioloogia piimaveisekarjades

#### Sissejuhatus

Piimakarjakasvatus on üks tähtsamaid põllumajandusharusid Eestis, moodustades 28% Eesti põllumajanduse kogutoodangust aastal 2018 (Maaeluministeerium, 2019). Hea tervisega lüpsilehmadelt saadud kõrge kvaliteediga toorpiim tagab Eesti piimatoodete konkurentsivõime. Üheks peamiseks haiguseks, mille tõttu piima kvaliteet halveneb, on mastiit (Hertl jt, 2014). Mastiit põhjustab suurt majanduslikku kahju, eeskätt suurenenud ravikulu, saamatajäänud piima ja lehmade enneaegse karjast praakimise tõttu (Hertl jt, 2014).

Mastiiti võivad põhjustada mitmed erinevad haigustekitajad, millest levinumad on *Staphylococcus aureus*, *Streptococcus uberis* ning *Escherichia coli*. Samas on mastiiditekitajate hulgas liike, mille tekitatud kahju on viimasel kümnendil seoses karjade ja tootmise intensiivistumisega suurenenud (Reksen jt, 2007). *Mycoplasma (M.) bovis* on mikroobiliik, mis võib lehmadel põhjustada mastiiti ning vasikatel liigese- ja kopsupõletikku (Bennet ja Jasper, 1978; Pfützner ja Schimmel, 1979).

*M. bovis*'e põhjustatud nakkustel on negatiivne majanduslik mõju piimaveisekasvatusele, sest nakatunud loomade heaolu halveneb. Nimetatud bakteri põhjustatud udaranakkuse tõttu väheneb lehmade piimatoodang, halveneb piima kvaliteet ja suureneb piima somaatiliste rakkude arv (Fox, 2012; Al-Farha jt, 2017).

Eesti Jõudluskontrolli Keskuse statistika järgi oli Eestis 2018. aastal ligikaudu 85 200 lüpsilehma, kellest 81 821 (96%) lehma kuulus vabatahtlikku jõudluskontrollisüsteemi. Keskmise 305-päeva piimatoodang ühe lehma kohta oli 2018. aastal jõudluskontrollis osalevates karjades 9785 kg. Karjad on aasta-aastalt suurenenud, keskmiselt oli nimetatud aastal karjas 160 lüpsilehma (Eesti Jõudluskontrolli Keskus, 2019).

Sõltumata pikaajalistest teadmistest *M. bovis*'e olemasolu ja ülemaailmse levimuse kohta, on selle haigustekitaja põhjustatud udaranakkuse

epidemioloogias mitmeid aspekte, mis vajaksid selgitamist. Näiteks ei ole uuritud tegureid, mis mõjutavad *M. bovis*'e udaranakkusest iseeneselikku tervistumist. Samuti on vähe uuritud *M. bovis*'e põhjustatud udaranakkuse riskitegureid ja karjasisest levimust, sh levikuteid. Nende asjaolude väljaselgitamine tooks uusi teadmisi nakkuse ohjamisel.

## Kirjanduse ülevaade

*M. bovis* kuulub *Mollicutes*'e bakterite perekonda. Need on rakukestata bakterid, kellele on omane parasitne eluviis (Razin, 1997; Rosengarten jt, 2000). Loomadel on enim levinud liigiks *M. bovis*, kuid veiste haigestumust võivad esile kutsuda ka *M. bovigenitalium*, *M. californicum*, *M. bovirhinis*, *M. alkalescens* ja *M. dispar* (Nicholas ja Ayling, 2003; Fox, 2012; Gioia jt, 2016). Veistel põhjustab *M. bovis* sagedamini udaranakkust (González ja Wilson, 2003), kuid esineda võib ka kopsupõletik igas vanuses veistel (Maunsell jt, 2011). Harvemini põhjustab bakter veiste aborte (Doig, 1981), meningiiti (Stipkovits jt, 1993), keskkõrva põletikku (Francoz jt, 2004; Foster jt, 2009), keratokonjunktiviiti (Alberti jt, 2006) ja nahaaluseid abstsesse ning liigesepõletikke (Kinde jt, 1993).

*M. bovis*'e peamised patogeensed omadused on peremeesorganismi rakkudele kinnitumine ja rakku sisenemine, immuunsüsteemi mõjutamine ja võime koloniseeruda kudedesse (Maunsell jt, 2011; Fox, 2012). Kõik nimetatud omadused võimaldavad sellel bakteril vältida peremeesorganismi immuunvastust ja põhjustada kroonilisi infektsioone (Buchenau jt, 2010; Fox, 2012).

*M. bovis*'e nakkus tuuakse tavaliselt karja subkliiniliselt haigete loomadega (Fox jt 2003; Gonzalez ja Wilson, 2003; Filioussis jt, 2007; Fox 2012). Kuna *M. bovis* liigitatakse nakkavaks udarapatogeeniks, võib haigustekitaja nakatunud loomadelt tervetele üle kanduda lüpsitoimingute käigus (Ruegg ja Erskine, 2015). Nakkuse ülekandumine võib nakatunud loomade kehaeritistega saastunud keskkonna või toidu vahendusel toimuda ka kaudselt, kusjuures saastunud piim on ka nakkuse ülekandeteeks vasikatele (Butler jt, 2000; Fox jt, 2005; Justice-Allen jt, 2010; Maunsell jt, 2011).

*M. bovis*'e nakkuse olemasolu kinnitamiseks karjas saab jahutipiima analüüsida mükoplasmavastaste antikehade (Nielsen jt, 2015; Petersen jt, 2016; Parker jt, 2017), bakteri DNA (Pinho jt, 2013; Bauman jt, 2018) või elusbakterite suhtes (Olde Riekerink jt, 2006; McDonald jt, 2009).

Elusa haigustekitaja tuvastamine jahutipiimas annab kinnitust, et karjas on vähemalt üks *M. bovis*'t piimaga eritav lehm (Biddle jt, 2003). *M. bovis*'e antikehade suhtes positiivne jahutipiim näitab haigustekitaja esinemist karjas. Karjasest haigustekitaja levimust ei saa jahutipiima analüüsil täpselt tuvastada ning nakatunud loomad tuleb kas individuaalproove või grupiproove uurides kindlaks teha (Fox jt, 2005).

*M. bovis*'e karjadevaheline levimus varieerub riikide lõikes. Taanis läbiviidud uuringutes leiti, et 7,2% uuritud jahutipiimadest olid *M. bovis*'e antikehade suhtes positiivsed (Nielsen jt, 2015). Arede jt (2016) poolt korraldatud aastase kestusega uuringus leiti, et *M. bovis*'e antikehade levimus piimaveisekarjades varieerus 1,5–5,2% vahel. Murai jt (2014) tuvastas Ameerika Ühendriikides ühe karja 1210 lehma üldpiimaproove (nelja udaraveerandi koondproovid) uurides, et 2,8% loomadest olid mükoplasmadega nakatunud.

*M. bovis*'e põhjustatud mastiit ei allu antibiootikumiravile (Maunsell jt, 2011), mistõttu soovitatakse tekitaja suhtes positiivsed loomad liigitada püsivalt nakatunuteks ning nad võimalusel karjast praakida. Kui praakimine ei ole kohe võimalik, tuleb nakatunud loomad teistest loomadest eraldada ja lüpssta viimasena (Pfützner ja Sachse, 1996; Gonzalez ja Wilson, 2003; Punyapornwithaya jt, 2011). Parim viis *M. bovis*'e nakkuse ennetamiseks on bioohutusreeglite järgimine (Maunsell jt, 2011). Hea lüpsihügieen hoiab ära *M. bovis*'e udaranakkuse ülekandumise lüpsitoimingute käigus (Fox jt, 2005; Bradley jt, 2012). Vasikate hulgas aitavad nakkuse ülekandumist ohjata head pidamistingimused, väike asustustihedus loomagruppides ning vasikatele joodetava toorpiima pastöriseerimine (Butler jt, 2000; Nicholas, 2011). Vaktsineerimine ei ole selle haiguse tõrjes tulemusi andnud. Eksperimentaaluurimustes on võrreldes laudatingimustes tehtud katsetega küll saadud paremaid tulemusi, kuid vaktsiinide toimet nakkuse ennetamiseks ei ole tõestatud (Chima jt, 1981; Boothby jt, 1987; Maunsell jt, 2009; Soehnen jt, 2011).

## Uurimistöö eesmärgid

Käesoleva uurimistöö eesmärg oli analüüsida *M. bovis*'e põhjustatud udaranakkuse epidemioloogiat piimaveisekarjades. Spetsiifilised eesmärgid olid:

1. Selgitada *M. bovis*'e udaranakkuse karjasisene levimus ja dünaamika Eesti piimaveisekarjades (artikkel I ja II);
2. Selgitada *M. bovis*'e esinemus veiste ternespiimas ja kliiniliste mastiitide korral (artikkel II);
3. Leida seoseid *M. bovis*'e udaranakkuse, piimatoodangu, piima koostise ja lehma udartervise näitajate vahel (artikkel I ja III)
4. Iseloomustada veiste kliinilisest mastiidist ja vasikate hingamisteedest isoleeritud *M. bovis*'e genotüüpe (artikkel II);
5. Hinnata *M. bovis*'e udaranakkusest tervenemist kinnisperioodi jooksul (artikkel III).

## Materjal ja metoodika

Väitekirja kahes esimeses teadusartiklis uuriti *M. bovis*'e karjasisest levimust ning selle mõju piimatoodangule ja udartervisele.

*M. bovis*'e udaranakkuse karjasisese levimuse hindamiseks (artikkel I) koguti 2014. aastal ühest Eesti piimaveisekarjast kõikidelt lehmadel üldpiimaproovid ( $n = 522$ ). Piimast määrati rasva- ja proteiinisaldus, soomaatiliste rakkude arv ning karbamiidisisaldus. Lisaks registreeriti andmed lehmade piimatoodangu, vanuse ning laktatsioonipäevade arvu kohta. Andmed analüüsiti lineaarse regressioonanalüüsiga, kasutades statistikaprogrammi STATA IC 10 (StataCorp, College Station, TX).

*M. bovis*'e udaranakkuse karjasisese levimuse muutust ajas uuriti 2017–2018. aastal (artikkel II), mil lehma üldpiimaproovid koguti neljast mükoplasma mastiiti põdevast piimaveisekarjast kolm korda kolmekuulise intervalliga. Esimesest karjast võeti kokku 1533, teisest 4549, kolmandast 1427 ja neljandast 2835 üldpiimaproovi. *M. bovis*'e esinemuse hindamiseks ternespiimas võeti kõikidelt katseperioodi jooksul poeginud lehmadel üldpiimaproovid ( $n = 1264$ ) esimese poegimisjärgse lüpsi käigus. Piimaproovid võeti ka katseperioodi jooksul kliinilisse mastiiti haigestunud lehmade udaraveeranditest ( $n = 707$ ). Proovide mikrobioloogiliseks analüüsiks segati lehmade üldpiimaproovid koondproovideks. Sarnaselt talitati ternespiimaproovide ja kliinilise mastiidi korral võetud piimaproovidega. Katsealustest karjadest koguti proovid ka vasikate hingamiselditest, et kindlaks teha karjas leviva *M. bovis*'e genotüübi võimalik ülekandumine lehmade ja vasikate vahel. Karjasisese levimuse analüüsiks kasutati statistikaprogrammi R (© The

R Foundation), kus Bayesiani statistika meetodeid kasutades arvutati levimushinnang.

Väitekirja kolmandas teadusartiklis hinnati udaraveerandite kinnisperioodiaegset tervistumist *M. bovis*'e udaranakkusest ühes Eesti piimaveisekarjas. Katses osales 133 lehma, kellelt võeti kinnijäämisel igast udaraveerandist piimaproov mikrobioloogiliseks analüüsiks (n = 510). Samadelt lehmadel koguti piimaproovid (n = 491) ka esimesel poegimisjärgsel päeval. Lineaarsete regressioonimudelitega uuriti tuvastatud udarapatogeenide ja udaraveerandi piimaproovist määratud haptoglobiini sisalduse vahelisi seoseid kinnijätmisel ning pärast poegimist. Statistiline andmeanalüüs tehti programmiga STATA IC 10 (StataCorp, College Station, TX).

Kõikides uuringutes tuvastati *M. bovis*'e olemasolu piimas reaallaja polümeraasi ahelreaktsiooniga (PCR) (Mastitis 4B, DNA Diagnostic, Risskov, Taani). *M. bovis*'e isoleerimiseks vasikate hingamisteedest ja kliinilise mastiidi proovidest kasutati mükoplasmaspetsiifilisi söötmeid ning isoleeritud mikroobide olemasolu proovis kinnitati PCR-meetodiga. Veiste kliinilise mastiidi piimaproovidest ja vasikate hingamiselunditest isoleeritud *M. bovis* genotüüpiseeriti tuumagenoomi multilokaalse järjestuse tüpiseerimise kaudu (*core genome multilocus sequence typing*, cg-MLST).

## Tulemused ja arutelu

Esimese uuringu tulemusena selgus, et *M. bovis*'e udaranakkuse karjasisene levimus oli 17,2% (95% usaldusväärsus 14,1–20,8), mida võib lugeda mõõdukaks levimuseks. Bakteri esinemine piimaproovides ei sõltunud lehma laktatsioonist ega laktatsioonipäevade arvust. *M. bovis*'e udaranakkus mõjutas lehma piimatoodangut oluliselt – nakatunud lehmade päeva piimatoodang oli keskmiselt 3 kg (p-väärtus 0,007) võrra madalam võrreldes loomadega, kellel bakteri DNA-d ei tuvastatud. Lehmadel, kes olid nakatunud *M. bovis*'ega, oli logaritmiliselt teisendatud somaatiliste rakkude arv piimas 0,8 ühikut (p-väärtus < 0,001) kõrgem võrreldes haigustekitaja suhtes negatiivsete loomadega. Lisaks oli nimetatud bakteri suhtes positiivsete lehmade piima rasvasisaldus 0,05 ühikut (p-väärtus 0,035) ja karbamiidisisaldus 15,6 ühikut (mg/l) (p-väärtus 0,002) väiksem võrreldes bakteriga mitternakatunud lehmade piimaga. Seevastu *M. bovis*'e positiivsetel lehmadel piima proteiinisaldus ei erinenud oluliselt mitternakatunud lehmade piima proteiinisaldusest.

Need tulemused näitavad, et varjatud *M. bovis*'e udaranakkusel on märkimisväärne mõju lehma piimatoodangule ja -kvaliteedile.

Teise uuringu tulemusena leiti, et *M. bovis*'e karjasisene levimus varieerus karjade lõikes. Keskmine levimushinnang jäi karjade esimesel testimisel 0,7–7,4% vahele. Teada on, et *M. bovis*'e udaranakkuse levimus on kõrgem suurtes piimaveisekarjades (USDA-APHIS, 2008). Loomade tihe paigutus ja üleasustus on *M. bovis*'e udaranakkuse riskitegur, mille puhul nakkuse levik toimub ka erinevate vanusegruppide vahel (Fox jt, 2005; Justice-Allen jt, 2010; Maunsell jt, 2011). *M. bovis*'e levimus lehmade ternespiimas oli kõikides karjades süiski väike, jäädes keskmiselt 1,7–4,7% vahele. Seetõttu võib oletada, et lehmade ternespiima individuaalse jootmise korral on haigustekitaja ülekandumine vasikatele ternespiimaga vähetõenäoline. *M. bovis*'e levimus kliinilise mastiidi korral võetud piimaproovides oli karjade lõikes keskmiselt 3,7–11,0%, millest võib järeldada, et endeemiliselt nakatunud piimaveisekarjades põhjustab *M. bovis* harva kliinilist mastiiti.

Vasikate hingamiseldidest, ternespiimaproovidest ja kliinilist mastiiti põdevate lehmade piimaproovidest pärit *M. bovis*'e genotüübid olid enamjaolt karjasiseselt sarnased. See viitab haigustekitaja vanusegruppidevahelisele ülekandele karja sees, mis võib toimuda otsese kontaktina vanusegruppide vahel või erinevate ülekandefaktorite, näiteks loomatalitajate vahendusel. Ka nimetatud patogeeni saastunud piim võib olla ülekandeteeks täiskasvanud veiste ja vasikate vahel.

Kolmanda uuringukäigus leiti, et *M. bovis*'e teituvastatud 86,7% udaraveerandi piimaproovidest esimesel lüpsil pärast poegimist, mis lehma kinnijätmisel olid selle patogeeni suhtes positiivsed. Selle haigustekitaja põhjustatud udaranakkus ei allu kinnisperioodiaegsele antibiootikumiravile (Ruegg ja Erskine, 2015). Kuigi udarakude on kinnisperioodi ajal ebasoodne keskkond bakterite paljunemiseks, võib nimetatud patogeen tungida rakkudesse ja püsida seal nakatamisvõimelisena kogu kinnisperioodi. Samuti on *M. bovis*'e eritumine piimaga vahelduv (Biddle jt, 2003), mistõttu ei saa väita, et lehmade udaraveerandid tervistusid *M. bovis*'e nakkusest. Haptoglobiin on akuutse faasi proteiin, mille sisaldus piimas suureneb udarakoe kahjustuse tagajärjel. Uurimistulemused näitasid, et *M. bovis*'e udaranakkus ei suurendanud oluliselt piima haptoglobiinisisaldust. Varasemaid uuringuid *M. bovis*'e udaranakkuse ja paikse põletikureaktsiooni vahel ei ole tehtud. Seetõttu võiksid järgnevad

katsed selgitada seoseid piima haptoglobiini kui lokaalse põletikunäitaja ja *M. bovis*'e udaranakkuse vahel.

## Järeldused

*M. bovis*'e udaranakkused mõjutavad negatiivselt lehma piimatoodangut ja -kvaliteeti ning nakatunud lehmade udaratervis on selle nakkuse korral halvem. Igas karjas oleks seetõttu soovitatav selgitada mükoplasma olemasolu ja udaranakkuse tuvastamisel tõrjemeetmeid rakendada.

*M. bovis* udaranakkuse karjasisene levimus oli 17,2% novembris 2014 tehtud uuringus, kus analüüsiti ühe karja 522 lakteeriva lehma üldpiimaproove qPCR meetodiga. *M. bovis*'e levimus uuringualustes karjades varieerus kuuekuulise katseperioodi jooksul aastatel 2017–2018, kuid oli uuringuperioodi jooksul üldiselt väike ja kõikum. *M. bovis*'e eritamine piimaga võib sõltuda nakkuse epideemia faasist, mistõttu on soovitatav loomade korduv uurimine nakkuse ulatuse hindamiseks.

Endeemiliselt nakatunud piimaveisekarjades oli *M. bovis*'e levimus ternespiimas väike ja võib oletada, et nakkuse ülekande tõenäosus vasikatele ternespiima vahendusel oli tühine. Kuna vasikatelt ja ternespiimast isoleeritud *M. bovis*'e genotüübid olid karjasiseselt sarnased, annab see tunnistust nakkuse karjasisesele vanuserühmade vahelisele ringlusele. Edasistes uuringutes oleks vaja selgitada *M. bovis*'e erinevate võimalike ülekandeteede tähtsust.

*M. bovis*'e väikese levimusega veisekarjades ei ole nimetatud haigustekitaja peamiseks kliinilise mastiidi põhjustajaks, sest *M. bovis*'e põhjustatud kliinilise mastiidi esinemus oli väike.

Suurem osa (86,7%) kinnijätmisel *M. bovis*'e suhtes positiivsetest udaraveeranditest olid pärast poegimist selle patogeeni suhtes negatiivsed. Edasistes uuringutes tuleks täiendavalt uurida, kas ja millisel määral tervistuvad *M. bovis*'e udaranakkusega lehmad kinnisperioodi jooksul. Samuti ei leitud olulist seost haigustekitaja esinemise ja udaraveerandi piimaproovide haptoglobiini sisalduse vahel enne kinnijätmist või pärast poegimist. *M. bovis*'e udaranakkuse ja udara paikse põletikureaktsiooni vahelisi seoseid tuleb veel edasistes katsetes uurida.

Edaspidised mükoplasma seotud uuringud võiksid hinnata *M. bovis*'e udaranakkuse dünaamikat pikema ajaperioodi jooksul, et mõista selle levimuse muutumist karjas. Samuti tuleks analüüsida mastiidikontrolli programmide mõju nakkuse karjasisesele levimusele. Täiendavaid uuringuid oleks vaja ka mükoplasma karjasiseste ülekandeteede selgitamiseks.

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## Within-herd prevalence of intramammary infection caused by *Mycoplasma bovis* and associations between cow udder health, milk yield, and composition

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### ABSTRACT

Subclinical mastitis is one of the major health problems in dairy herds due to decreased milk production and reduced milk quality. The aim of this study was to examine the within-herd prevalence of subclinical intramammary infection caused by *Mycoplasma bovis* and to evaluate associations between *M. bovis* and cow daily milk yield, udder health, and milk composition. Individual cow composite milk samples (n = 522) were collected from all lactating dairy cows in 1 Estonian dairy farm in November 2014. Daily milk yield, days in milk, and parity were recorded. Collected milk samples were analyzed for somatic cell count, milk protein, fat, and urea content. The presence of *M. bovis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis* in the milk samples was confirmed by quantitative PCR analysis. The within-herd prevalence of *M. bovis* was 17.2% in the study herd. No association was observed between days in milk and parity to the presence of *M. bovis* in milk. According to linear regression analysis, the daily milk yield from cows positive for *M. bovis* was on average 3.0 kg lower compared with cows negative for *M. bovis*. In addition, the presence of *M. bovis* in milk samples was significantly associated with higher somatic cell count and lower fat and urea content compared with milk samples negative for *M. bovis*. In conclusion, subclinical *M. bovis* intramammary infection is associated with decreased milk yield and lower milk quality.

**Key words:** *Mycoplasma bovis* mastitis, dairy cow, prevalence, milk yield

### INTRODUCTION

Mastitis, which can be caused by different udder pathogens, is one of the major concerns in dairy herds because it causes economic losses to the industry due to lower milk production and reduced milk quality (Ruegg, 2012; Hertl et al., 2014). In addition, milk fat and protein concentration have been shown to decrease due to lipolysis and proteolysis in mastitic milk (Larsen et al., 2010; Vidanarachchi et al., 2015; Zhang et al., 2015).

The biggest effect on dairy herd milk quality and production arises from contagious mastitis pathogens such as *Staphylococcus aureus*, as well as *Streptococcus agalactiae* (Reksen et al., 2007; Paradis et al., 2010; Sørensens et al., 2010). *Mycoplasma bovis*, a bacterium lacking a cell wall from genus *Mycoplasma*, mainly causes IMI (Nicholas and Ayling, 2003; Maunsell et al., 2011). *Mycoplasma bovis* is usually classified as a contagious mastitis pathogen (USDA APHIS, 2008; Royster and Wagner, 2015). Transmission between animals occurs mainly at the milking time (Ruegg, 2012). *Mycoplasma bovis* usually causes subclinical or mild clinical IMI, which can progress to chronic mastitis. Severe clinical mastitis outbreaks may also develop (Bushnell, 1984; Pothmann et al., 2015; Ruegg and Erskine, 2015). *Mycoplasma bovis* mastitis is not treatable with antibiotics; therefore, the control strategies of *M. bovis* IMI are to keep the herd uninfected, and to segregate and cull infected cows (Fox et al., 2005; Royster and Wagner, 2015; Nicholas et al., 2016).

Traditionally, *M. bovis* has been identified using culture-based methods, but due to low sensitivity and a long incubation period, molecular diagnostic methods have become preferable during recent years (Dorman et al., 1983; Sachse et al., 2010; Gioia et al., 2016). Detection of *M. bovis* in bulk tank milk samples (BTMS) or cow composite milk samples (CMS) by using quantitative PCR (qPCR) allows an identification of udder pathogens, including *M. bovis*, rapidly, with high

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sensitivity, and without previous isolation of bacteria (Ghadersohi et al., 1997; Ghadersohi et al., 1999; Fox et al., 2005). Identification of infected herds is best made by analyzing BTMS. *Mycoplasma bovis* positive result indicates that the pathogen is introduced to the herd. However, the true within-herd prevalence cannot be estimated with only a positive BTMS result. Further identification of infected cows should be made by analyzing CMS (Fox et al., 2005).

Although mycoplasmas were identified in North America and Europe decades ago, the prevalence of *M. bovis* mastitis is not widely studied (Fox et al., 2005; Fox, 2012). The herd prevalence of *M. bovis* udder infection ranges between 0.9% in Australia and 1.5% in Belgium (Passchyn et al., 2012; Morton et al., 2014). According to a longitudinal study made in Israel, the number of *M. bovis*-positive dairy herds has increased annually from 2008 to 2014 (Lysnyansky et al., 2016). However, the within-herd prevalence of *M. bovis* mastitis is still not widely studied to this day. By knowing the within-herd prevalence and course of the disease, it could be possible to develop functional control programs and predict new outbreaks. In a study by Murai et al. (2014), a within-herd prevalence of *M. bovis* mastitis was 2.8% (n = 1,210). According to the Estonian Animal Recording Centre database, 19.6% of BTMS (n = 112), analyzed with the PCR method, were positive to *M. bovis* in 2013 (Estonian Animal Recording Centre, 2014).

To our knowledge, no studies are available about the associations between *M. bovis* mastitis and cow milk yield or milk composition. Research about this, however, would clarify the importance of *M. bovis* as an udder pathogen and as a cause of production losses.

The first objective of this study was to identify the within-herd prevalence of subclinical IMI caused by *M. bovis* by qPCR method. The second aim was to find associations between subclinical *M. bovis* infection, cow daily milk yield, SCC, and milk composition.

## MATERIALS AND METHODS

### Characteristics of the Study Herd

The milk samples were collected from one large, loose-housing dairy herd in Estonia in November 2014. The study herd included 611 dairy cows, of which 89% were Estonian Holstein, and 11% were Estonian Red breed cows. Cows were milked twice a day in the 2 × 12 parallel milking parlors. The average 305-d milk yield was 9,916 kg and bulk milk SCC ranged between 259,000 and 358,000 cells/mL in 2014. *Mycoplasma bo-*

*vis* was previously detected in BTMS and cow CMS of single cows with clinical mastitis by PCR in 2011.

### Collection and Analysis of Composite Milk Samples

The CMS of all 525 lactating dairy cows were collected once during the routine milk recording in November 2014. The daily milk yield of each cow was measured by using a calibrated milk meter (Tru-Test Limited, Auckland, New Zealand). Parity and DIM of all lactating dairy cows were recorded.

All milk samples were preserved with bronopol and transported to the milk laboratory of the Estonian Animal Recording Centre in Tartu. In the laboratory, milk fat (%), protein (%), urea (mg/L), and SCC (× 1,000/mL) were analyzed with accredited methods using the automatic analyzer Combifoss 6000 FC (Hillerød, Denmark).

After analysis, 1.5 mL of each milk sample was collected and transported to the Estonian University of Life Sciences. Cows with visible signs of clinical mastitis (n = 3) were excluded from the study, and 2 cows without production data were excluded only from the regression analysis. All milk samples (n = 522) were stored at -18°C for further analysis.

### qPCR Analysis of Milk Samples

A commercial qPCR test kit Mastit4B (DNA Diagnostic A/S, Risskov, Denmark) was used for qPCR analysis to detect bacterial DNA directly from the milk samples.

The oligos of the Mastitis 4B are designed to detect DNA of *Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, and *M. bovis*. After thawing, the milk samples were vortexed and from each sample, 500 µL of milk was used for DNA extraction before PCR analysis according to the instructions ([http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction\\_protocol\\_M4B\\_2017.04.26.pdf](http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction_protocol_M4B_2017.04.26.pdf)) from the manufacturer (DNA Diagnostic, Risskov, Denmark). The PCR mixture consisted of 15 µL of the qPCR Master Mix and 5 µL of purified DNA. The real-time PCR instrument thermal cycler Stratagene Mx3005P (Agilent Technologies Inc., Santa Clara, CA) was used for amplification. The amplification conditions were as follows: 95°C for 1 min, 1 cycle; 95°C for 5 s and 60°C for 25 s, 40 cycles. Cycle threshold (Ct) values were reported for all samples. For all bacteria identified in the analysis, a Ct value of ≤37.0 was considered a positive result. The assay included controls for the validation of each run including negative DNA extraction controls, internal amplification standard

**Table 1.** Different combinations of detected bacteria in pathogen-positive milk samples

Pathogen	Number of positive samples (%)
<i>Streptococcus agalactiae</i>	75 (37.5)
<i>Mycoplasma bovis</i>	30 (15.0)
<i>Staphylococcus aureus</i>	14 (7.0)
<i>Streptococcus uberis</i>	3 (1.5)
<i>Mycoplasma bovis, Streptococcus agalactiae</i>	39 (19.5)
<i>Streptococcus agalactiae, Staphylococcus aureus</i>	17 (8.5)
<i>Mycoplasma bovis, Staphylococcus aureus, Streptococcus agalactiae</i>	15 (7.5)
<i>Mycoplasma bovis, Staphylococcus aureus</i>	6 (3.0)
<i>Streptococcus agalactiae, Staphylococcus aureus, Streptococcus uberis</i>	1 (0.5)
Total	200 (100)

(positive PCR controls), and nontemplate control. The assay was validated on both bacterial strains and milk samples by DNA Diagnostic. According to the internal validation protocol of the laboratory, the sensitivity and specificity of the test kit Mastitis 4B is 100% when tested directly on a bacterial colony.

### Statistical Analyses

A causal diagram was drawn for variables to evaluate their causal associations and identify any confounders. Dependent variables were milk yield (kg), SCC ( $\times 1,000/\text{mL}$ ), milk protein, fat percentages, and milk urea content (mg/L). Cow parity and DIM were considered to be confounders according to the causal diagram. A linear regression model was chosen for estimating the associations between udder pathogens and milk yield, SCC, and milk compositions. The distribution of dependent variables was checked graphically. To achieve normal distribution, natural logarithm transformation was used for SCC, the square root was taken from the milk fat content, and an inverse scale was used for milk protein content.

The PCR test results were dichotomized for each bacterium as either presence or absence by using the cut-off values ( $\leq 37.0$ ) set by the manufacturer. Associations between the presence of 3 mastitis pathogens (*M. bovis*, *Staph. aureus*, and *Strep. agalactiae*) cow parity, and DIM were assessed using the  $\chi^2$  test. Due to low number of qPCR-positive *Strep. uberis* CMS, the cow infection status of that pathogen was not added to the risk factor models. Statistical significance was set at  $P \leq 0.05$ .

Multivariable models were composed separately for each production indices serving as outcome variables. In multivariable models, cow parity (categorized into 1, 2, and  $\geq 3$  lactations) and DIM (categorized as 1–90, 91–200, and  $\geq 201$  DIM) were inserted to control for confounding effects. Dichotomized results (yes = 1, no = 0) of *M. bovis*, *Staph. aureus*, and *Strep. agalactiae* were included to assess the association with the out-

come variable. Interaction terms were tested for significance to see whether the combined effect of 2 mastitis pathogens differs from the sum of the individual effects of the pathogens tested (Dohoo et al., 2009). Assumptions of the equal variance of the outcome in all levels of predictor variables and normal distribution of the residuals were checked graphically (Dohoo et al., 2009).

The STATA IC 10 (StataCorp, College Station, TX) software was used for statistical analyses.

## RESULTS

### Descriptive Statistics

Out of 522 cow CMS, 38.3% ( $n = 200$ ) contained DNA from at least one targeted bacterial species. The DNA of one detected bacterial species was found in 61% ( $n = 122$ ), 2 species in 31% ( $n = 62$ ), and 3 species were detected simultaneously in 8% ( $n = 16$ ) of pathogen-positive milk samples. *Mycoplasma bovis* alone was detected in 15% ( $n = 30$ ) of pathogen-positive milk samples and in combination with *Strep. agalactiae* in 19.5% ( $n = 39$ ) of pathogen-positive milk samples. The most prevalent bacterial species was *Strep. agalactiae* alone, presented in 37.5% ( $n = 75$ ) of pathogen-positive milk samples. The different combinations of udder pathogens in pathogen-positive cow CMS are present in Table 1.

### Prevalence of Udder Pathogens

The within-herd prevalence of *M. bovis* was 17.2% ( $n = 90$ ; 95% CI 14.1–20.8). *Streptococcus agalactiae* had the highest within-herd prevalence of 28.4% ( $n = 148$ ; 95% CI 24.5–34.2). The within-herd prevalence of *Staph. aureus* was 10.2% ( $n = 53$ ; 95% CI 7.7–13.1). *Streptococcus uberis* was found only in 5 CMS with prevalence of 1% (95% CI 0.3–2.2).

The presence of *M. bovis* or *Staph. aureus* was not significantly associated with DIM. The probability of detecting *Strep. agalactiae* in milk samples was higher

**Table 2.** Distribution of detected mastitis pathogens (alone or in combination) in composite milk samples of 520 dairy cows according to lactation stage and parity

Item	Number of cows	<i>Mycoplasma bovis</i> positive, no. (%)	<i>Staphylococcus aureus</i> positive, no. (%)	<i>Streptococcus agalactiae</i> positive, no. (%)
<b>DIM</b>				
0-90	133	23 (17.3)	15 (11.3)	25 (18.8)
91-200	170	35 (20.6)	15 (8.8)	53 (31.2)
≥201	217	32 (14.7)	23 (10.6)	70 (32.2)*
<b>Parity</b>				
1 Lactation	220	29 (13.2)	18 (8.2)	58 (26.4)
2 Lactations	159	30 (18.9)	13 (8.2)	49 (30.8)
≥3 Lactations	141	31 (22.0)	22 (15.6)*	41 (29.1)

\*Statistically significant in  $\chi^2$  test ( $P < 0.05$ ) by DIM or lactation number.

( $P < 0.05$ ) in dairy cows in late lactation stage (>201 DIM) compared with the first 3 mo of lactation. There was a significantly higher risk of detecting *Staph. aureus* in milk samples from older cows (≥3 lactations) compared with ≤2 lactation dairy cows (Table 2).

**Associations Between Detected Mastitis Pathogens and Milk Yield, Somatic Cell Count, and Milk Composition**

The presence of *M. bovis* DNA in milk samples was associated with a lower (−3.0 kg) daily milk yield compared with dairy cows without *M. bovis* DNA in the milk samples. The daily milk yield was also lower (−4.0 kg) in dairy cows that tested positive for *Strep. agalactiae* compared with *Strep. agalactiae* negative dairy cows (Table 3).

The lnSCC was significantly higher in milk samples, in which DNA from *M. bovis*, *Staph. aureus*, and *Strep. agalactiae* was detected, compared with milk samples negative for these pathogens (Table 4).

In the CMS of cows positive for *M. bovis*, the milk fat and urea ( $P < 0.05$ ) content were lower compared

with the respective values of cows negative for *M. bovis*. *Mycoplasma bovis* was not significantly associated with milk protein content (Tables 5, 6, and 7). The presence of *Strep. agalactiae* in milk samples had a positive association with the milk fat content ( $P = 0.041$ ) and protein content ( $P = 0.034$ ; Tables 5 and 6).

**DISCUSSION**

**Prevalence of *Mycoplasma bovis* in Cow CMS Using qPCR**

The within-herd prevalence of subclinical *M. bovis* udder infection is not widely studied in European dairy herds. Most of the previous studies are focusing on clinical mastitis cases or on between-herd prevalence of *M. bovis* (Filioussis et al., 2007; Arcangioli et al., 2011; Radaelli et al., 2011; Passchyn et al., 2012). We identified a *M. bovis* within-herd prevalence of 17.2% in one dairy herd. Previously, Brown et al. (1990) identified a 5.7% (n = 1535) prevalence of mycoplasmas based on a study in one herd. According to the study of Brown et al. (1990), a higher within-herd prevalence of *M. bovis*

**Table 3.** Results of multivariable linear regression model of association between detected mastitis pathogens in cow composite milk samples and daily milk yield (kg) of dairy cows (n = 520)

Item	Number of cows	Coefficient	95% CI	P-value	Wald test
<i>Mycoplasma bovis</i> negative	430	0			
<i>Mycoplasma bovis</i> positive	90	−3.0	−5.2; −0.8	0.007	
<i>Staphylococcus aureus</i> negative	467	0			
<i>Staphylococcus aureus</i> positive	53	0.8	−1.8; 3.5	0.546	
<i>Streptococcus agalactiae</i> negative	372	0			
<i>Streptococcus agalactiae</i> positive	148	−4.0	−5.9; −2.2	<0.001	
1 Lactation	220	0			<0.001
2 Lactations	159	3.7	1.9; 5.5	<0.001	
≥3 Lactations	141	3.3	1.4; 5.3	0.001	
<90 DIM	133	0			<0.001
91-200 DIM	170	−4.4	−6.5; −2.3	<0.001	
≥201 DIM	217	−13.6	−15.6; −11.6	<0.001	
Intercept		36.0	34.2; 37.9	<0.001	

**Table 4.** Results of multivariable linear regression model of association between SCC in cow composite milk samples and mastitis pathogens (n = 520)

Item	Number of cows	Coefficient <sup>1</sup>	95% CI	P-value	Wald test
<i>Mycoplasma bovis</i> negative	430	0			
<i>Mycoplasma bovis</i> positive	90	0.8	0.5; 1.1	<0.001	
<i>Staphylococcus aureus</i> negative	467	0			
<i>Staphylococcus aureus</i> positive	53	0.9	0.5; 1.3	<0.001	
<i>Streptococcus agalactiae</i> negative	372	0			
<i>Streptococcus agalactiae</i> positive	148	0.6	0.3; 0.8	<0.001	
1 Lactation	220	0			<0.001
2 Lactations	159	0.1	-0.2; 0.4	0.459	
≥3 Lactations	141	0.6	0.3; 0.8	<0.001	
<90 DIM	133	0			<0.001
91-200 DIM	170	0.1	-0.2; 0.4	0.399	
≥201 DIM	217	0.5	0.2; 0.8	<0.001	
Intercept		3.7	3.4; 4.0	<0.001	

<sup>1</sup>Estimates are in logarithmic scale.**Table 5.** Results of multivariable linear regression model of association between milk fat in cow composite milk samples and mastitis pathogens (n = 520)

Item	Number of cows	Coefficient <sup>1</sup>	95% CI	P-value	Wald test
<i>Mycoplasma bovis</i> negative	430	0			
<i>Mycoplasma bovis</i> positive	90	-0.05	-0.1; -0.004	0.035	
<i>Staphylococcus aureus</i> negative	467	0			
<i>Staphylococcus aureus</i> positive	53	-0.1	-0.07; 0.05	0.732	
<i>Streptococcus agalactiae</i> negative	372	0			
<i>Streptococcus agalactiae</i> positive	148	0.05	0.002; 0.09	0.041	
1 Lactation	220	0			0.021
2 Lactations	159	-0.06	-0.1; -0.01	0.011	
≥3 Lactations	141	-0.05	-0.1; -0.001	0.046	
<90 DIM	133	0			<0.001
91-200 DIM	170	-0.03	-0.08; 0.02	0.233	
≥201 DIM	217	0.08	0.04; 0.1	0.001	
Intercept		2.0	1.95; 2.04	<0.001	

<sup>1</sup>Estimates are on a square root scale.**Table 6.** Results of multivariable linear regression model of association between milk protein in cow composite milk samples and mastitis pathogens (n = 520)

Item	Number of cows	Coefficient <sup>1</sup>	95% CI	P-value	Wald test
<i>Mycoplasma bovis</i> negative	430	0			
<i>M. bovis</i> positive	90	0.001	-0.005; 0.007	0.706	
<i>Staphylococcus aureus</i> negative	467	0			
<i>Staph. aureus</i> positive	53	0.002	-0.006; 0.009	0.674	
<i>Streptococcus agalactiae</i> negative	372	0			
<i>Strep. agalactiae</i> positive	148	-0.006	-0.01; -0.0004	0.034	
1 Lactation	220	0			0.282
2 Lactations	159	0.003	-0.002; 0.008	0.214	
≥3 Lactations	141	0.004	-0.002; 0.009	0.190	
<90 DIM	133	0			<0.001
91-200 DIM	170	-0.02	-0.02; -0.01	<0.001	
≥201 DIM	217	-0.04	-0.05; -0.04	<0.001	
Intercept		0.3	0.30; 0.31	<0.001	

<sup>1</sup>Estimates are on an inverse scale (negative estimate means higher content of protein).

IMI was found in our study. However, we collected milk samples only once and from a single dairy herd by using a high-sensitivity qPCR method to detect major udder pathogens from the CMS (Koskinen et al., 2010; Murai et al., 2014; Nyman et al., 2016). Therefore, the results of this study describe the within-herd prevalence of *M. bovis* only at the single point in time in one specific herd.

Cow CMS are used to detect especially subclinical IMI when it is difficult to identify the infected udder quarter. However, milk originating from noninfected udder quarters may lower the sensitivity in IMI pathogen detection, due to a dilution effect (Reyher and Dohoo, 2011). In this study, we used commercial qPCR analysis to detect 4 mastitis pathogens in cow CMS simultaneously. A high sensitivity of the PCR method to detect udder pathogens causing IMI is reported, also when cow CMS are used (Friendship et al., 2010; Koskinen et al., 2010; Murai et al., 2014; Nyman et al., 2016).

A carry-over of the udder pathogen DNA may occur when CMS are collected with automated milk meters. Milk from a previously milked cow is mixed with milk from the cow currently being milked, leading to false-positive PCR test results (Løvendahl et al., 2010; Mahmmod, 2015). In conventional milking systems, the probability of carry-over is evaluated to range from 2 to 3.5% (Løvendahl and Bjerring, 2006; Løvendahl et al., 2010). The carry-over effect may be associated with the Ct-value cut-off (Mahmmod et al., 2014). Lower cut-off values in discriminating positive and negative test results lower the sensitivity of the test, but reduce the number of false-positive test results that may occur due to carry-over (Mahmmod et al., 2014). The cut-off value was set to be  $\leq 37.0$  in our study, which should be low enough to rule out most of the false-positive results and hence decrease the probability that contaminated

samples are classified as positive (Mahmmod et al., 2014).

#### Associations Between Cow *Mycoplasma bovis* Infection and Milk Yield and Milk Composition

To our knowledge this is the first study evaluating associations between subclinical *M. bovis* IMI, cow milk yield, SCC, and composition, when cow CMS are used. We identified no significant relation between co-infection with udder pathogens and cow milk yield, SCC, or milk composition. This may be due to relatively low numbers of samples with multiple pathogens detected and should be controlled in further studies using larger number of samples.

The presence of *M. bovis* bacterial DNA in CMS was associated with an average of a 3.0 kg lower daily milk yield compared with cows negative for *M. bovis*. The negative effect of *Strep. agalactiae* or *Staph. aureus* subclinical IMI on milk yield was already discovered decades ago (Keefe et al., 1997; Reksen et al., 2007). In the present study, a negative association between presence of bacterium in subclinically infected cow CMS and milk yield was detected for *Strep. agalactiae* but not for *Staph. aureus*. Even though the number of *M. bovis* positive dairy cows was low in our study, a significant relation between *M. bovis* and cow daily milk yield was identified. As far as we know, no research has been published about the association between subclinical *M. bovis* IMI and milk yield. Therefore, this study provides essential knowledge about the economic effect of *M. bovis* on dairy farm production.

A significant association was observed between *M. bovis* and milk SCC level after controlling for the presence of other mastitis pathogens, parity, and DIM. We found that in CMS positive for *M. bovis*, the lnSCC was on average 0.8 units higher compared with milk

**Table 7.** Results of multivariable linear regression model of association between milk urea (mg/L) in cow composite milk samples and mastitis pathogens (n = 520)

Item	Number of cows	Coefficient	95% CI	P-value	Wald test
<i>Mycoplasma bovis</i> negative	430	0			
<i>M. bovis</i> positive	90	-15.6	-25.6; -5.6	0.002	
<i>Staphylococcus aureus</i> negative	467	0			
<i>Staph. aureus</i> positive	53	-6.0	-18.2; 6.3	0.339	
<i>Streptococcus agalactiae</i> negative	372	0			
<i>Strep. agalactiae</i> positive	148	-7.8	-16.4; 0.7	0.072	
1 Lactation	220	0			<0.001
2 Lactations	159	-3.8	-12.2; 4.7	0.379	
$\geq 3$ Lactations	141	-16.8	-25.7; -7.9	<0.001	
<90 DIM	133	0			<0.001
91–200 DIM	170	28.2	18.7; 37.7	<0.001	
$\geq 201$ DIM	217	17.1	8.1; 26.2	<0.001	
Intercept		159.4	150.8; 168.0	<0.001	

samples negative for this pathogen. It is speculated that the SCC of cows positive for *M. bovis* is higher in most of the cows, but not all of them (Ghadersohi et al., 1999; Pinho et al., 2013). Even though in our study there was an association between *M. bovis* IMI and cow SCC, using only cow SCC status in mastitis control programs may lead to an omission of some *M. bovis* positive dairy cows (Ghadersohi et al., 1999; Pinho et al., 2013).

In our study, the subclinical *M. bovis* udder infection was negatively associated with some of the milk composition parameters. The milk fat and urea content in *M. bovis*-positive cows was lower than in *M. bovis*-negative cows. Spontaneous lipolysis during the IMI leads to lower milk fat content and changes in milk fat composition (Forsbäck et al., 2010). Urea is formed in the liver from ammonium and absorbed to the milk from the blood in a stable form (Hayton et al., 2012). However, many factors influence the milk urea content, such as altered DMI, or the lack of rumen degradable protein and energy in feed ratio (Rezamand et al., 2007; Hayton et al., 2012). Therefore, it is not possible to make strong conclusions about the causality of *M. bovis* subclinical IMI and milk urea content.

Intramammary infection usually causes a decrease in milk protein levels (Rezamand et al., 2007). In this study, we did not find a significant association between the presence of *M. bovis* in milk samples and milk protein content. Further studies using a larger sample size are needed to confirm the associations between subclinical *M. bovis* IMI and milk yield as well as milk composition parameters, making it possible to estimate the economic effect of *M. bovis* IMI and effects on cow well-being.

### CONCLUSIONS

This study identified the within-herd prevalence of subclinical *Mycoplasma bovis* IMI of 17.2% by testing CMS of 522 lactating dairy cows using qPCR. Dairy cows infected with *M. bovis* had higher SCC, produced less milk, and had lower milk fat and urea content compared with *M. bovis*-negative dairy cows. Further studies should evaluate the associations between *M. bovis* and milk yield and milk quality in a larger number of herds. Our findings underline the importance to apply control measures of *M. bovis* mastitis to reduce economic losses due to lower milk yield and milk quality caused by subclinical udder infection of *M. bovis*.

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## Dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds

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### ABSTRACT

We aimed to identify the dynamics of the within-herd prevalence of *Mycoplasma (M.) bovis* intramammary infection (IMI) in four dairy herds, estimate prevalence of *M. bovis* in colostrum and clinical mastitis cases and compare *M. bovis* strains from calves' respiratory and cow clinical mastitis samples. Within a six-month study period, cow composite milk samples (CMS) were collected three times during routine milk recording, first milking colostrum samples from all calving cows and udder quarter milk samples from clinical mastitis cases. Calf respiratory samples were collected from calves with respiratory disease. Pooled milk samples were analysed for *M. bovis* with the Mastitis 4B polymerase chain reaction (PCR) test kit (DNA Diagnostic A/S). Prevalence estimates were calculated with Bayesian framework in R statistical programme. cg-MLST was used for *M. bovis* genotyping. In Herd I and II first testing *M. bovis* IMI within-herd prevalence (95 % credibility interval (CI)) was 4.7 % (2.9; 6.8) and 3.4 % (2.3; 4.6), changing to 1.0 % (0.1; 1.7) and 0.8 % (0.1; 1.4) in Herd I and 0.4 % (0.0; 0.7) in Herd II at the next samplings. In Herd III and IV first testing *M. bovis* IMI within-herd prevalence was 12.3 % (9.7; 15.2) and 7.8 % (6.2; 9.5), changing to 4.6 % (3.0; 6.4) and 3.2 % (1.9; 4.8) in Herd III and to 2.8 % (1.9; 3.8) and 4.9 % (3.6; 6.4) in Herd IV at the next samplings. The estimated prevalence of *M. bovis* in colostrum ranged between 1.7 % (0.2; 2.8) and 4.7 % (2.7; 7.1) and in clinical mastitis cases between 3.7 % (1.7; 6.4) and 11.0 % (7.5; 15.2) in the study herds. *M. bovis* strains isolated from cows and calves clustered within herds indicating possible transmission of *M. bovis* between dairy cows and calves. Prevalence of *M. bovis* in colostrum and clinical mastitis cases as well as the within-herd prevalence of *M. bovis* IMI was low in endemically infected dairy herds.

### 1. Introduction

Mastitis is one of the most common diseases in dairy cows, causing economic losses to the dairy industry due to lower milk yield and reduced milk quality (Hertl et al., 2014). *Mycoplasma (M.) bovis* is an emerging udder pathogen causing intramammary infection (IMI) in dairy herds (Nicholas and Ayling, 2003; Maunsell et al., 2011). *M. bovis* is usually classified as a contagious mastitis pathogen, and it is transmitted between dairy cows mainly during milking procedures (USDA APHIS, 2008; Ruegg, 2012; Royster and Wagner, 2015). *M. bovis* may cause subclinical or mild clinical mastitis, which can progress to chronic infection. However, severe clinical mastitis outbreaks may also occur (Bushnell, 1984; Pothmann et al., 2015; Ruegg and Erskine,

2015). *M. bovis* frequently colonises the respiratory mucosa and may cause respiratory disease (Rosengarten et al., 2000; Maunsell and Donovan, 2009). Otitis media and arthritis caused by *M. bovis* also occurs in calves (Maunsell et al., 2011). In *M. bovis*-positive dairy herds, transmission of *M. bovis* infection to calves is thought to occur via unpasteurised raw milk from cows shedding *M. bovis* (Maunsell and Donovan, 2009). Heat treatment or pasteurisation of cow colostrum and raw milk have been used to reduce and eliminate possible pathogens, including *M. bovis* (Stabel et al., 2004; Godden et al., 2006). However, to our knowledge, no studies have evaluated the presence or prevalence of *M. bovis* in cow colostrum as a possible risk factor for infection transmission from dairy cows to calves.

Mycoplasmas were identified in North America and Europe decades

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ago (Fox et al., 2005; Fox, 2012), but the within-herd prevalence of *M. bovis* IMI has not been widely studied. A within-herd prevalence of *M. bovis* IMI of 2.8 % (n = 1210, 95 % CI 1.9; 3.7) and 0.6 % (95 % CI = 0.0, 1.4) were detected in two herds in a study conducted by Murai et al. (2014). Additionally, *M. bovis* IMI within-herd prevalence of 17.2 % (n = 522, 95 % CI 14.1; 20.8) was identified in one Estonian dairy herd in November 2014 (Timonen et al., 2017). However, there has been no published longitudinal or repeated studies evaluating the within-herd prevalence of *M. bovis* IMI, which would benefit the understanding of the disease patterns. Polymerase chain reaction (PCR) detects DNA of udder pathogens from bulk tank milk samples and cow composite milk samples (CMS) rapidly, with higher sensitivity compared to bacteriologic cultivation, and without preceding bacteriology (Ghadersohi et al., 1999; Fox et al., 2005; Koskinen et al., 2010). PCR should be an adequate analysis for the estimation of *M. bovis* IMI within-herd prevalence using pooled cow CMS, as it is a cost-effective diagnostic tool with adequate sensitivity in large dairy herds (Murai et al., 2014).

Molecular epidemiologic methods are used nowadays to identify infection sources, transmission and spread of udder pathogens within and between dairy farms (Gurjar et al., 2012). Multi-locus sequence typing (MLST) (Lysnyansky et al., 2016) and core genome MLST (cg-MLST) (Haapala et al., 2018) are successfully used to identify strains of *M. bovis* isolated from cattle with mastitis. Genotyping the *M. bovis* isolates enables a comparison between *M. bovis* strains at herd and animal level and provides a useful tool to identify different transmission routes of *M. bovis* infections.

The objective of this study was to evaluate dynamics of within-herd prevalence of *M. bovis* IMI in four Estonian dairy herds by using pooled cow composite milk samples. The second objective of this study was to evaluate the prevalence of *M. bovis* IMI in cow colostrum and clinical mastitis cases by using pooled samples. Additionally, the study aimed to compare the genotypes of *M. bovis* strains isolated from cows' clinical mastitis udder quarter milk and calves' respiratory samples.

## 2. Materials and methods

### 2.1. Study design and herd characteristics

This repeated cross-sectional study was performed between September 2017 and October 2018 in four large Estonian dairy herds. Herd characteristics are given in Table 1. All the study herds were previously identified to be positive for *M. bovis* based on the bulk tank milk PCR analysis at least six months before the beginning of the study. Bulk tank milk PCR Ct-values ranged between 30.2 and 39.4 in the study herds at the first study month.

A six-month study period was set for each study herd. For the evaluation of the within-herd prevalence of *M. bovis* IMI, cow CMS was collected from all lactating cows in all study herds three times at three-month intervals during routine milk recording. Cow composite colostrum samples were collected from all cows calving during the study period. Additionally, udder quarter-level milk samples were collected from all cows that suffered clinical mastitis during the study period. To identify the strains of *M. bovis* in calves, tracheobronchial lavage (TBL), nasal swab or deep nasopharyngeal swab samples were collected from five to ten calves with respiratory disease symptoms in the last study month of the herd. The sample collection scheme is described Fig. 1.

### 2.2. Collection of milk samples

Cow CMS were collected into 20 ml sterile plastic tubes during regular milk recording. All CMS were preserved with bronopol and transported to the Estonian University of Life Sciences. Cow colostrum samples were collected at the first milking after calving by pre-trained farm personnel. Colostrum samples (40 ml) were frozen at the farms and transported to the Estonian University of Life Sciences. When

clinical mastitis was diagnosed in cow (changes in milk appearance and/or swollen and/or painful udder quarter), an aseptically collected udder quarter milk sample was collected by pre-trained farm personnel. The teat end was cleaned with 70 % ethanol swabs and allowed to dry. After discarding a few streams of milk, samples (2–4 ml) were collected into sterile 10-ml plastic tubes. Samples were frozen and transported to the Estonian University of Life Sciences.

### 2.3. Pooling of milk samples

Based on the PCR analysis of bulk tank milk in the first study month, the within-herd prevalence of *M. bovis* IMI was expected to be low in the study herds, and cow CMS, colostrum and udder quarter milk samples were pooled at the Estonian University of Life Sciences. For each pooling, two millilitres of individual milk sample (CMS, colostrum or udder quarter milk sample) was used. At the first sampling, five cows' CMS were pooled. At the second and third samplings, twenty cows' CMS were pooled in herds III and IV and 100 cows' CMS in Herds I and II. Pools of 20 and 100 cows' CMS were used due to expected low within-herd prevalence of *M. bovis* IMI based on first study month bulk tank milk PCR analysis. Colostrum pools were combined from 40 cows' and 10 cows' colostrum samples as the prevalence of *M. bovis* in colostrum was expected to be low. Ten udder quarter milk samples originating from clinical mastitis cases were pooled for 1 pool. Udder quarter milk samples collected from clinical mastitis cases and udder quarter milk sample pools are referred to as "clinical mastitis cases" and "pools of clinical mastitis cases", respectively, in the text from here on.

### 2.4. Collection of calf respiratory samples

TBL, nasal swab or deep nasopharyngeal swab samples were collected from calves (aged 1–6 months) with clinical signs of respiratory disease once from all dairy herds. A calf health scoring chart (University of Wisconsin-Madison, 2020) was used to choose the calves for sampling. Calves having at least 1 present clinical symptom (nasal discharge, coughing, high body temperature ( $\geq 39.3^\circ\text{C}$ ), difficult breathing or dyspnoea in auscultation) with scores of 1 were chosen for sampling. For TBL sampling, the calf was restrained by an assistant, calf nostrils were cleaned with gauze and the sample was collected with a sterile and flexible double sampling catheter. The sampling catheter was first introduced to the upper respiratory tract through the ventral meatus of the nose. The catheter was introduced further until the caudal pharynx was reached with felt resistance. Further, the catheter was introduced to the trachea during the inspiratory phase of the respiratory cycle. When the double catheter was inside the trachea, the inner catheter was pushed through the silicone plug of the outer catheter and into the lungs as far as possible (Nikunen et al., 2007). Forty millilitres of sterile sodium chloride solution (0.9 %) was inserted into the respiratory tract and immediately aspirated. Collected fluid was placed in 10-ml plastic tubes and cooled. Calf nasal swab samples were collected with sterile nasal swabs (Sterile Dry Swab, Copan Diagnostics Inc., USA), inserted circa 5–10 cm to nostrils. For collection of calves' deep pharyngeal swab samples, calf nostrils were cleaned with gauze. Samples were collected with sterile nasopharyngeal swab (Dryswab®, United Kingdom) inserted approximately 25 cm into the upper respiratory tract. Nasal and nasopharyngeal swabs were placed to Friis broth for transportation and cooled. After collection of calves' respiratory samples, they were transported to Estonian Veterinary and Food Laboratory in Tartu for immediate bacterial cultivation.

### 2.5. Laboratory analysis

#### 2.5.1. PCR analysis

Frozen CMS, colostrum and clinical mastitis udder quarter milk sample pools were transported to the DNA Diagnostic laboratory in Risskov, Denmark for PCR analysis. Mastitis 4B, a commercial

**Table 1**  
Key parameters of the four study herds in the year 2018.

	Herd 1	Herd 2	Herd 3	Herd 4
First detection of <i>Mycoplasma bovis</i> in bulk tank milk	January 2017	January 2017	November 2014	October 2017
Ct-value of first study month bulk tank milk PCR analysis <sup>1</sup>	34.8	39.4	30.2	33.8
<b>Dairy cows</b>				
Avg. number of cows	591	1,633	552	1,035
Housing system of cows	Free stall	Free stall	Free stall	Free stall
Milking system	2 × 12 Parallel milking parlour	Carousel	2 × 12 Parallel milking parlour	2 × 12 Parallel milking parlour
Herd avg. 305-day milk yield (kg/cow)	10,502	10,404	9,751	10,095
Herd avg. bulk tank SCC <sup>2</sup>	177,000	158,000	391,000	343,000
Separate grouping of cows with clinical mastitis	Yes	Yes	Yes	Yes
Milking order of clinical mastitis group	Last	Last	Last	Last
Separate grouping of cows with high SCC <sup>3</sup>	No	No	Yes	No
Milking order of high SCC group	Not relevant	Not relevant	Penultimate	Not relevant
<b>Calves ≤ 6 months of age</b>				
Avg. number of calves	158	422	164	260
Separation from dam	Immediately after birth	Immediately after birth	Immediately after birth	Immediately after birth
Time spent in individual calf box after birth	7 days	7 days	7 days	7 days
Duration of colostrum feeding	2-4 days since birth	2-4 days since birth	4 days since birth	2-4 days since birth
Feeding after colostrum period	Milk powder	Milk powder	Milk powder (females) Raw milk (bull calves)	Milk powder
Pasteurising colostrum	No	No	No	No

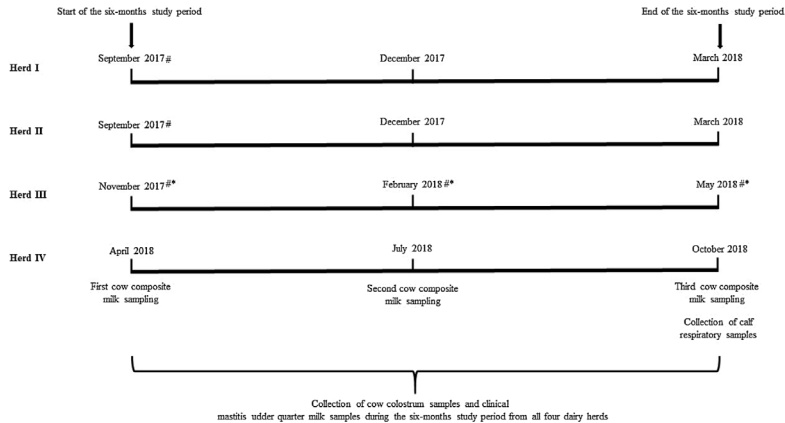
<sup>1</sup> Cycle threshold value (Ct-value) of the polymerase chain reaction analysis of bulk tank milk in the first study month of the herd.

<sup>2</sup> Somatic cell count per ml of milk.

<sup>3</sup> Cows with somatic cell count < 200 000 cells/ml are grouped separately.

quantitative PCR test kit (DNA Diagnostic A/S, Denmark) was used for PCR analysis to detect bacterial DNA of *M. bovis* directly from the milk samples. Cow colostrum sample pools were analysed in 2 rounds. First, pools of 40 cows' colostrum were analysed with PCR and then pools of 10 cows' colostrum samples belonging to *M. bovis* PCR positive pools of 40 cows' colostrum. Further PCR analysis on the individual animal level was not performed in any of the milk samples. The oligos of the Mastitis 4B are designed to detect DNA of *Staphylococcus aureus*, *Streptococcus*

*agalactiae*, *Streptococcus uberis* and *M. bovis*. After thawing, the milk samples were vortexed and from each sample, 500 µl of milk was used for DNA extraction before PCR analysis according to the manufacturer's instructions (DNA Diagnostic, Risskov, Denmark). The PCR mixture consisted of 15 µl of the PCR Master Mix and 5 µl of purified DNA. The thermal cycler Stratagene Mx3005 P real-time PCR instrument (Agilent Technologies Inc., Santa Clara, CA) was used for amplification. The amplification conditions were as follows: 95 °C for 1 min, 1 cycle; 95 °C



**Fig. 1.** Sample collection scheme for four dairy herds participating in the study between September 2017 and October 2018. A six-month study period was set for each herd. Cow composite milk samples were collected three times from all dairy herds. Cow colostrum samples and clinical mastitis under quarter milk samples were collected during the whole six-month study period in each herd. Calf respiratory samples were collected once at the last study month of the herd. Detection time of *Mycoplasma bovis* PCR-positive clinical mastitis under quarter pools is represented as # and detection time of *Mycoplasma bovis* PCR-positive colostrum pools as \*.

for 5 s and 60 °C for 25 s, 40 cycles. Cycle threshold (Ct) values were reported for all samples. For all bacteria identified in the analysis, a Ct value of  $\leq 37.0$  was considered a positive result. The assay included controls for the validation of each run, including negative DNA extraction controls, internal amplification standard (positive PCR controls) and non-template control. The assay was validated on both bacterial strains and milk samples by the DNA Diagnostic. According to the internal validation protocol of the laboratory, the sensitivity and specificity of the Mastitis 4B test kit is 1.0 %, when tested directly on a bacterial colony.

#### 2.5.2. Isolation of *M. bovis*

Cultivation of *M. bovis* PCR positive pools of clinical mastitis cases ( $n = 19$ ) and calves' respiratory samples was done in the Estonian Veterinary and Food Laboratory in Tartu. An accredited cultivation procedure was performed for clinical mastitis milk samples with a cultivation of an aliquot of 0.1 ml of milk sample in mycoplasma-specific Hayflick agar plates (Pfitzner and Sachse, 1996). The aliquot of milk sample was evenly distributed over the plates by sterile spatula and allowed to dry before incubation as described by Olde Riekerink et al. (2006). An isolation method of mycoplasmas from tissue material was used for cultivation of calves' respiratory samples. An aliquot of 0.01 ml of calves' respiratory samples in Friis transport broth were evenly inoculated in Friis agar (Yeary and Nietfeld, 2002; Gabinaitiene et al., 2011). Both Hayflick agar and Friis agar plates were incubated in a humid atmosphere at 37 °C under 5 % CO<sub>2</sub> concentration for 7 days. Daily microscopic observation for fried egg shaped mycoplasma colonies was performed. Suspected colonies were analysed with PCR for *M. bovis* identification (Francouz et al., 2012).

#### 2.5.3. Core-genome multilocus sequence typing

For cg-MLST analysis, *M. bovis* isolates from calves' respiratory samples ( $n = 5$ ) and clinical mastitis cases ( $n = 5$ ) were selected from Herds I and III. DNA isolation and WGS was performed at Finnish Food Authority as described by Haapala et al. (2018), with minor modifications. Nextera® Flex DNA Library Preparation kit (Illumina, USA) was used for library preparation with DNA inputs of 100 ng per library, and the libraries were sequenced on a MiSeq instrument (Illumina, USA) using MiSeq Reagent Kit v2 (500-bp) chemistry (Illumina, USA).

#### 2.6. Statistical analysis

The true within-herd prevalence of *M. bovis* IMI and the prevalence of *M. bovis* in cow colostrum and clinical mastitis cases was estimated based on PCR-positive cow CMS pools, colostrum pools and clinical mastitis sample pools. Within-herd prevalence of *M. bovis* IMI was calculated separately for each herd and sampling round. Pool size varied depending on the sampling time as described above. Prevalence of *M. bovis* in colostrum samples and in clinical mastitis cases was calculated separately for each herd within the six-month study period. Ten cows' colostrum samples were used for prevalence estimation in each herd.

Estimation of the true within-herd prevalence of *M. bovis* IMI and prevalence in colostrum and clinical mastitis cases based on pooled milk samples requires factors about test sensitivity (Se) and specificity (Sp) (Boelaert et al., 2000). Sensitivity is considered the most important parameter in the calculation of true prevalence based on pooled samples, because the objective is to identify the minimum proportion of truly infected animals at herd level (Murai et al., 2014). Test sensitivity (Se) was set to range uniformly between 77 % and 81.3 % in all pool sizes as no perfect Se estimate was available for pooled milk sample analysis with PCR. Lower test Se of 77 % was applied according to the study by Justice-Allen et al. (2011) describing Se of bulk tank milk PCR analysis for *M. bovis*. Higher limit of Se 81.3 % was adapted from Murai et al. (2014) studying within-herd prevalence of *M. bovis* IMI based on pooled CMS samples in different pool sizes. This range of test Se should

not exaggerate the power of PCR analysis to detect *M. bovis* DNA also in low amounts in pooled milk samples. In contrast, the objective of a whole herd test is not to gauge truly negative results (non-infected animals), and test Sp was not considered to violate the test results (Murai et al., 2014). Hence, no positive PCR results were considered false-positives. Test specificity (Sp) adapted from the manufacturer was set to be 99.95 % for all pool sizes.

Statistical programme R (The R Foundation®) was used for the calculation of prevalence estimates with package prevalence (Center for Burden and Risk Assessment, 2020) and rjags (Sourceforge, 2020) using model truePrevPools (R Package Documentation, 2020) for estimation of true prevalence from pooled samples (Boelaert et al., 2000; Speybroeck et al., 2012). This model estimates the true prevalence from the apparent prevalence in a Bayesian framework with credibility intervals (CI). The relationship between individual and pool test characteristics (Boelaert et al., 2000) is considered in the model. Bayesian estimation assumes population parameters to have an intrinsic probability distribution giving 95 % CI indicating 95 % chance of the true values lying between the limits of CI (Gardner, 2002). Uncertainty on unknown and variable parameters in diagnostic test characteristics is considered in the Bayesian model allowing a flexible combination of complex equations (Speybroeck et al., 2012). Prior distribution in this study was formed as beta posterior distribution, which is the conjugate prior to the proportion/probability parameter in a binomial distribution (Dohoo et al., 2014). Function betaExpert was used to calculate the parameters of a beta distribution based on expert information. For this, the best-guess prevalence was specified to vary between 0.1 % and 20.0 % with the most likely prevalence of 9.0 % specified. The calculated alpha and beta estimates of the beta distribution were used in setting the prior beta distribution in truePrevPools model.

### 3. Results

#### 3.1. Descriptive statistics

In total, 1,533 CMS were collected from Herd I, 4,549 CMS from Herd II, 1,427 CMS from Herd III and 2,835 CMS from Herd IV during the study. Within the study period, 1,264 colostrum samples were collected from four study herds. Four pools of ten cows' colostrum  $n = 28$  tested were positive for *M. bovis* in Herd III. *M. bovis* was not detected from pools of ten cows' colostrum in Herd I ( $n = 60$  tested), Herd II ( $n = 23$  tested) or Herd IV ( $n = 16$  tested). During the study period, 707 udder quarter milk samples were collected from clinical mastitis cases from the four study herds. Six *M. bovis*-positive pools of clinical mastitis cases originated from Herd I ( $n = 26$  tested), one *M. bovis*-positive pool of originated from Herd II ( $n = 20$  tested), and 13 *M. bovis*-positive pools of clinical mastitis cases originated from Herd III ( $n = 23$  tested). From Herd IV, seven pools of clinical mastitis cases were available, but none of these were *M. bovis*-positive in PCR analysis.

In total, 33 respiratory tract samples were collected from calves from four study herds. From the respiratory samples, five *M. bovis* isolates were identified. *M. bovis* isolates were identified from eight udder quarter milk sample pools from clinical mastitis cases (Table 2).

#### 3.2. The estimated within-herd prevalence and prevalence of *M. bovis* in colostrum and clinical mastitis samples

The estimated within-herd prevalence of *M. bovis* IMI in four study herds during the study is shown in Table 3. Over four farms, the estimated within-herd prevalence of *M. bovis* IMI ranged between 0.4 % (95 % CI 0.0; 0.7) and 12.3 (95 % CI 9.7; 15.2) during the study period.

In cow colostrum samples, the estimated prevalence of *M. bovis* ranged between 1.7 % (95 % CI 0.2; 2.8) and 4.7 % (95 % CI 2.7; 7.1) evaluated by pools of ten cows' colostrum samples (Table 4). The estimated prevalence of *M. bovis* in clinical mastitis cases in the study herds during the study ranged between 3.7 % (1.7; 6.4) and 11.0 %

**Table 2**

*Mycoplasma bovis* isolates from four dairy herds identified by cultivation on Hayflick agar at 37 °C and 10 % CO<sub>2</sub> and used for core genome multilocus sequence typing.

Herd	Number of samples taken	Sample type	Number of isolates
I	2	TBL <sup>1</sup>	1
	6	Clinical mastitis case <sup>2</sup>	1
	6	Nasal swab	0
II	2	Deep nasopharyngeal swab	0
	1	Clinical mastitis case <sup>2</sup>	0
	8	Nasal swab	4
III	13	Clinical mastitis case <sup>2</sup>	4
	15	Deep nasopharyngeal swab	0
IV	5	Clinical mastitis case <sup>2</sup>	0

<sup>1</sup> Tracheobronchial lavage.

<sup>2</sup> Udder quarter milk sample pool of 10 udder quarters deriving from clinical mastitis cases.

(7.5; 15.2) (Table 5).

### 3.3. Comparison of *M. bovis* strains

One calf respiratory isolate and cow clinical mastitis isolate originated from Herd I, whereas four calf respiratory isolates and 4 cow clinical mastitis isolates originated from Herd III (Fig. 2).

The cgMLST schema targets covered 58.2 % of the reference genome. From the 10 isolates in cg-MLST analysis, 527 cgMLST allele-called targets were extracted and compared with each other. *M. bovis* isolates from calves' respiratory samples and cows' milk samples clustered together within herds. The cow mastitis and calves' respiratory strains had allele differences of 5 in Herd I. In Herd III, mastitis and respiratory strains had allele differences of 1–5, with an exception in sample K27, which had an allele difference of 53 compared to other strains from the herd. Allele difference between Herd I and Herd III was at least 106 (Fig. 2).

## 4. Discussion

### 4.1. Within-herd prevalence of *M. bovis* intramammary infection

Within-herd prevalence of *M. bovis* was identified thrice at single time points with three-month intervals between milk recordings. The within-herd prevalence of *M. bovis* IMI varied between four dairy herds during a six-month study period but was generally low (0.4%–12.3%). The study herds were selected based on an *M. bovis* positive bulk tank milk sample without prior knowledge about the epidemic phase of the *M. bovis* IMI. Still, herds were infected at least six months before collection of first cow CMS and close to the start of the study. Hence, a relatively low within-herd prevalence could be suspected due to high BTM Ct-values indicating low amount of the pathogen secreted in milk.

**Table 3**

Within-herd prevalence of *Mycoplasma bovis* intramammary infection in study herds during a six-month study period evaluated by pooled cow composite milk samples.

Sampling	First sampling			Second sampling			Third sampling		
	n positive/ n total <sup>1</sup>	% <sup>2</sup>	95 % CI	n positive / n total <sup>1</sup>	% <sup>2</sup>	95 % CI	n positive / n total <sup>1</sup>	% <sup>2</sup>	95 % CI
Herd I	5 / 96	4.7	2.9; 6.8	0 / 5 <sup>3</sup>	1.0	0.1; 1.7	0 / 6 <sup>3</sup>	0.8	0.1; 1.4
Herd II	9 / 276	3.4	2.3; 4.6	0 / 15 <sup>3</sup>	0.4	0.0; 0.7	0 / 15 <sup>3</sup>	0.4	0.0; 0.7
Herd III	28 / 92	12.3	9.7; 15.2	10 / 25	4.6	3.0; 6.4	5 / 23	3.2	1.9; 4.8
Herd IV	31 / 187	7.8	6.2; 9.5	10 / 47	2.8	1.9; 3.8	22 / 47	4.9	3.6; 6.4
Pool size	5 cows			20 / 100 <sup>3</sup> cows			20 / 100 <sup>3</sup> cows		

<sup>1</sup> Number of udder pathogen positive pools / number of pools analysed.

<sup>2</sup> Estimated within-herd prevalence of *Mycoplasma bovis*.

<sup>3</sup> Pools of hundred cows.

**Table 4**

Prevalence of *Mycoplasma bovis* in cow colostrum in study herds during a six-month study period evaluated by pooled colostrum samples.

Herd	<i>Mycoplasma bovis</i> positive pools	Total number of pools analysed	% <sup>1</sup>	95 % CI
Herd I	0	23	2.8	0.2; 4.6
Herd II	0	60	1.7	0.2; 2.8
Herd III	4	28	4.7	2.7; 7.1
Herd IV	0	16	3.4	0.3; 5.6

<sup>1</sup> Estimated prevalence of *Mycoplasma bovis* in colostrum calculated by pools of ten colostrum samples.

**Table 5**

Prevalence of *Mycoplasma bovis* in clinical mastitis cases in study herds during a six-month study period evaluated by pooled udder quarter milk samples.

Herd	<i>Mycoplasma bovis</i> positive pools	Total number of pools analysed	% <sup>1</sup>	95 % CI
Herd I	6	26	5.9	3.7; 8.7
Herd II	1	20	3.7	1.7; 6.4
Herd III	13	23	11.0	7.5; 15.2
Herd IV	0	7	5.0	0.3; 8.5

<sup>1</sup> Estimated prevalence of *Mycoplasma bovis* in clinical mastitis cases calculated by pools of ten udder quarter milk samples.

In study Herds I and II, the within-herd prevalence was close to zero after the first CMS sampling whereas in the herds III and IV, *M. bovis*-positive cow CMS pools were identified during the whole study period. These different patterns might be due to different epidemic phases of the infection as well as strain differences and infection transmission possibilities in the farms. Also, an intermittent shedding of mycoplasma organism in cows with mycoplasma IMI could lead to these differences as described by Biddle et al. (2003). Additionally, some cows with mycoplasma IMI secrete a low concentration of mycoplasma organisms (10<sup>6</sup> cells/ml) in CMS (Biddle et al., 2003). Hence, the results of this study should be interpreted with caution. Further studies should evaluate the dynamics of *M. bovis* IMI since the time of infection and during a longer time-span to understand better the pattern of *Mycoplasma bovis* IMI.

### 4.2. The prevalence of *M. bovis* in colostrum and clinical mastitis cases

This is the first study identifying the prevalence of *M. bovis* in cow colostrum samples in large commercial dairy herds. The estimated prevalence of *M. bovis* in cow colostrum was low in all study herds, indicating that in endemically infected dairy herds, the shedding of *M. bovis* in colostrum is minimal. Additionally, the estimated prevalence of *M. bovis* in clinical mastitis cases ranged between 3.7 % and 11.0 % in the herds during the study. *M. bovis* being an infrequent cause of mastitis in endemically infected herds of this study is consistent with

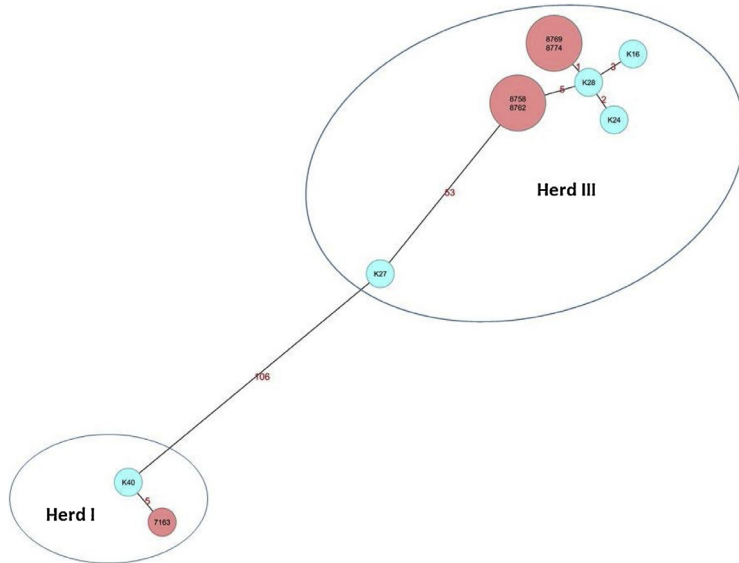


Fig. 2. Minimum spanning tree constructed from the core genome multilocus sequence typing allele profiles from *Mycoplasma bovis* strains isolated from cow clinical mastitis cases (turquoise circles) and calves' respiratory samples (red circles) from two dairy herds (Herds I and III). Sample ID-numbers are given inside the red and turquoise circles. Allele differences between isolates are shown in red (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the findings of Vähänikkilä et al. (2019), who collected 3,268 udder quarter milk samples from clinical and subclinical mastitis cases and identified only 51 cows with *M. bovis* clinical mastitis in recently infected herds during a two-year study period. Even though the prevalences were low in CMS, colostrum and clinical mastitis cases, waste milk is not advisable to feed to calves in order to inhibit the transmission of pathogen between age groups. Ideally, colostrum should be pasteurised before feeding to calves.

#### 4.3. Prevalence estimation using pooled milk samples

We estimated the prevalence of *M. bovis* using pooled CMS, colostrum and clinical mastitis milk samples. Pooled testing to estimate individual-animal prevalence is justified if assumed prevalence is < 10 % (Cowling et al., 1999), even though pooling causes a potential decrease in sensitivity compared to individual-animal testing (Christensen and Gardner, 2000). We used pools of five to 100 cows' milk samples in prevalence estimations as the *M. bovis* prevalence in study herds was expected to be low based on the bulk tank milk PCR analysis. Murai et al. (2014) calculated a Se of 81.3 % for the estimation of *M. bovis* IMI within-herd prevalence by using first pools of 100 cows' CMS analysed with PCR followed by PCR analysis of 50 cows' CMS and finally individual cow CMS cultivation. In this study, Se for the calculation of prevalence estimations was lowered to a range between 77 % and 81.3 % to adjust the prevalence estimates to imperfect Se when using pooled milk samples. Calculation of prevalence estimates was based on a Bayesian framework, which is an exact method and allows the parameters to be unknown (Cowling et al., 1999). Hence, the power of PCR analysis to detect low amounts of *M. bovis* DNA from large pools is

considered in this study and is not exaggerated.

#### 4.4. Comparison of *M. bovis* strains

The allele difference between *M. bovis* strains from two herds was large, indicating circulation of different strains in the two herds. In general, calves' respiratory and cows' clinical mastitis *M. bovis* strains had low allele differences within herds. An exception was one clinical mastitis *M. bovis* strain, which differed from other *M. bovis* strains originating from calves' respiratory samples and clinical mastitis udder quarter milk samples in Herd III. New *M. bovis* strains may enter the dairy herds with subclinically infected and imported animals (Fox, 2012). The farm created a beef unit housed close to a dairy youngstock barn in 2016, and direct contact was possible between beef cattle and dairy heifers over the fence on pasture, possibly causing the transmission of new *M. bovis* strains to the dairy unit.

Clustered *M. bovis* strains originating from animals in different ages within the same herd refers to circulation of pathogen between calves and cows. *M. bovis* is commonly thought to be transmitted between cows and calves through contaminated milk (Maunsell and Donovan, 2009; Fox, 2012). In this study, calves were fed with individual cow colostrum originating from cows from the same study herd followed by milk from post-partum cows for two to four days after birth and then milk replacer until weaning. During the first week of calves' life, the calves were immediately separated from their dams and housed in individual boxes in the same barn. Environmental or air-borne transmission of *M. bovis* is possible as *M. bovis* may survive in cool and humid conditions for long periods (Pfützner, 1984) and air-borne transmission of *M. bovis* has been described (Nicholas, 2011). Hence, transmission of

*M. bovis* between cows and calves is possible via air-borne or indirect contact during the first week of calves' life. Additionally, direct transmission between calves in group pens after the first week of calves' life and indirect transmission of *M. bovis* through farm-personnel's contaminated hands and equipment is possible. Further studies should evaluate the specific transmission routes of *M. bovis* between adult dairy cows and calves.

## 5. Conclusions

The estimated average within-herd prevalence of *M. bovis* IMI during a six-month study period varied between four dairy herds ranging between 0.4% and 12.3%. The within-herd prevalence of *M. bovis* IMI and the prevalence of *M. bovis* in cow colostrum and in clinical mastitis cases was low in endemically infected dairy herds. Herd-specific *M. bovis* strains isolated from calves and cows indicate a circulation of the pathogen between cattle of different ages. Further studies should evaluate the dynamics of *M. bovis* IMI during a longer period, starting since the introduction of the disease. Additionally, further molecular epidemiologic studies should evaluate the transmission routes of *M. bovis* within dairy herds.

## Declaration of Competing Interest

None

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## Elimination of selected mastitis pathogens during the dry period

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### ABSTRACT

We aimed to evaluate the elimination of 4 different mastitis pathogens, *Streptococcus agalactiae*, *Mycoplasma bovis*, *Staphylococcus aureus*, and *Streptococcus uberis*, from infected udder quarters during the dry period using quantitative PCR. The second purpose of this study was to evaluate the association between milk haptoglobin (Hp) concentration and the presence of udder pathogens (*Strep. agalactiae*, *Staph. aureus*, *M. bovis*, and *Strep. uberis*) in udder quarter milk samples before and after dry period. Aseptic udder quarter milk samples (n = 1,001) were collected from 133 dairy cows at dry off and at the first milking after calving from 1 large dairy herd. Bacterial DNA of *Strep. agalactiae*, *Staph. aureus*, *Strep. uberis*, and *M. bovis* in the udder quarter milk samples was identified with commercial quantitative PCR analysis Mastitis 4B (DNA Diagnostic A/S, Risskov, Denmark). Milk Hp concentration (mg/L) was measured from udder quarter milk samples. The elimination rates during the dry period for *M. bovis*, *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* were 86.7, 93.6, 96.2, and 100.0%, respectively. The new IMI rate was 3.0% for *M. bovis*, 2.9% for *Staph. aureus*, 2.4% for *Strep. agalactiae*, and 3.1% for *Strep. uberis*. The milk Hp concentration was significantly higher in udder quarter milk samples with blood and in samples positive for *Strep. agalactiae* at dry off and for *Staph. aureus* postcalving. Elevated milk Hp concentration was not associated with the presence of *M. bovis* in the udder quarter milk samples. In conclusion, elimination of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* during the dry period was high; the elimination of *M. bovis* from infected udder quarters was lower, but probably spontaneous. Additionally, milk Hp concentration may be used as a marker for udder inflammation when combined with the bacteriological results at dry off and postpartum.

**Key words:** udder pathogens, dry period, elimination, haptoglobin

### INTRODUCTION

Mastitis is one of the major concerns in dairy herds because it causes economic losses to the dairy industry due to lower milk yield and reduced milk quality (Hertl et al., 2014). Cow mammary glands are more susceptible to the invasion of udder pathogens at dry off and around calving. Natural defense mechanisms, such as lactoferrin, leukocytes, and keratin plug at the teat end, are present in the mammary gland, inhibiting the invasion and growth of udder pathogens during the dry period. To enhance the elimination of udder pathogens from infected udder quarters, dry cow antibiotic therapy alone or together with internal teat-sealants is used at dry off (Bradley and Green, 2004). Usually, treatment against *Streptococcus agalactiae* is effective during the dry period, but recovery from *Staphylococcus aureus* is more difficult; however, some strain differences affect the elimination of *Staph. aureus* in dry cows (Dingwell et al., 2006). Dry cow therapy is considered ineffective in the elimination of *Mycoplasma bovis* from infected udder quarters (Ruegg and Erskine, 2015). Traditionally, the presence of udder pathogens before and after calving has been identified with culture-based methods, but molecular methods, such as PCR, have become more common in detecting udder pathogens from milk samples worldwide (Koskinen et al., 2010).

The mammary environment during the dry period is not advantageous for udder pathogens, and acute clinical mastitis rarely occurs in dry cows (Bradley and Green, 2004). However, subclinical IMI during the dry period serves as a risk factor for clinical mastitis after calving (Bradley and Green, 2004). Mastitis-causing bacteria entering the mammary gland via the teat canal trigger a local inflammatory response and increase the level of acute phase proteins in milk (Pyörälä, 2003). Haptoglobin (Hp), one of the acute phase proteins, is mainly produced in the liver, but local production of Hp in mammary gland occurs as Hp is released from the damaged epithelial cells and neutrophils of udder

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tissues (Hiss et al., 2004). Milk Hp concentration can be used as a marker for the detection of udder inflammation (Pyörälä, 2003), as both clinical and subclinical IMI induce elevated milk Hp concentrations (Nielsen et al., 2004; Kalmus et al., 2013).

The elimination rates of *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* during the dry period (Bradley et al., 2015) and the associations between these udder pathogens and milk Hp concentration (Eckersall et al., 2006; Pyörälä et al., 2011) have been previously described. No published studies have examined the elimination of *M. bovis* during the dry period. Additionally, associations between *M. bovis* and local inflammatory response in the mammary glands measured through milk Hp concentration have not been studied to our knowledge. The aim of our study was to evaluate the elimination of *Strep. agalactiae*, *Staph. aureus*, *M. bovis*, and *Strep. uberis* from infected udder quarters during the dry period using quantitative PCR (qPCR) method. The second aim of our study was to evaluate the association between milk Hp concentration and the presence of udder pathogens (*Strep. agalactiae*, *Staph. aureus*, *M. bovis*, and *Strep. uberis*) in udder quarter milk samples at dry off and postcalving.

## MATERIALS AND METHODS

### Study Design

Cow aseptic udder quarter milk samples were collected once at dry off and from the first milking after calving between November 2014 and May 2015 from 1 Estonian dairy herd. All the cows that were dried off and calved during that period were sampled.

All the collected udder quarter milk samples ( $n = 1,001$ ) were analyzed with qPCR for the detection of bacterial DNA of *Staph. aureus*, *Strep. agalactiae*, *M. bovis*, and *Strep. uberis*. Based on the results of the qPCR analysis, the elimination rates and new infection rates were calculated. After qPCR analysis, milk Hp concentration (mg/L) was measured from all collected udder quarter milk samples to evaluate the associations between the presence of udder pathogens and milk Hp concentration.

### Characteristics of the Study Herd

The milk samples were collected from 1 Estonian large loose-housed dairy herd from northeastern Estonia. *Mycoplasma bovis* was previously identified in bulk tank milk samples and cow composite milk samples in clinical mastitis cases in 2013. The study herd included 611 dairy cows, of which 89% were Estonian Holstein

and 11% Estonian Red. Cows were milked twice per day in a  $2 \times 12$  parallel milking parlor. The average 305-d milk yield was 9,916 kg, and the bulk milk SCC ranged between 259,000 and 358,000 cells/mL in 2014. All cows were treated with cloxacillin-based dry cow antibiotic product (Noroclox DC, 600 mg, Norbrook Laboratories Limited, Newry, UK) at dry off. The length of the dry period ranged between 37 and 94 d (median 65 d). Cow parity, DIM, and the length of the dry period were recorded from the database of Estonian Livestock Performance Recording Ltd. (Tartu, Estonia).

### Collection of Udder Quarter Milk Samples

Cow udder quarter milk samples were collected at dry off and at the first milking postpartum. Before collection, the teat end was cleaned with 70% ethanol swabs and allowed to dry. After discarding a few streams of milk, samples (2 to 4 mL) were collected into sterile 10-mL plastic tubes. Milk samples were stored at  $-18^{\circ}\text{C}$  and transported to the DNA Diagnostic A/S laboratory for further analysis.

### qPCR Analysis of Udder Quarter Milk Samples

Bacterial DNA from *M. bovis*, *Staph. aureus*, *Strep. agalactiae*, and *Strep. uberis* was detected by commercial quantitative qPCR test kit Mastitis 4B (DNA Diagnostic A/S). The oligos of the Mastitis 4B are designed to detect DNA of *Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, and *M. bovis*. After thawing, the milk samples were vortexed and from each sample and 500  $\mu\text{L}$  of milk was used for DNA extraction before PCR analysis according to the manufacturer's instructions (DNA Diagnostic A/S, [http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction\\_protocol\\_M4B\\_2017.11.01.pdf](http://dna-diagnostic.com/files/Downloads/Mastit4/Instruction_protocol_M4B_2017.11.01.pdf)). The PCR mixture consisted of 15  $\mu\text{L}$  of the qPCR Master Mix and 5  $\mu\text{L}$  of purified DNA. The real-time PCR instrument thermal cycler Stratagene Mx3005P (Agilent Technologies Inc., Santa Clara, CA) was used for amplification. The amplification conditions were  $95^{\circ}\text{C}$  for 1 min for 1 cycle, and  $95^{\circ}\text{C}$  for 5 s and  $60^{\circ}\text{C}$  for 25 s for 40 cycles. Cycle threshold (Ct) values were reported for all samples. For all bacteria identified in the analysis, a Ct value of  $\leq 37.0$  was considered a positive result. The assay included controls for the validation of each run, including negative DNA extraction controls, internal amplification standard (positive PCR controls), and nontemplate control. The assay was validated on both bacterial strains and milk samples by the DNA Diagnostic.

### Determination of Elimination Rate During the Dry Period

According to the results of PCR analysis, udder quarters were classified as positive (P) or negative (N) for detected udder pathogens both at dry off and postpartum. Based on different infectious status combinations, elimination and new IMI rates were classified and calculated for each detected udder pathogen separately. Principles of classification of elimination and new IMI rates of the mastitis pathogen during the dry period were calculated following Dufour and Dohoo (2012) as

$$\begin{aligned} \text{Elimination} &= (P_{\text{dry}} \text{ and } N_{\text{pp}}) / [(P_{\text{dry}} \text{ and } N_{\text{pp}}) \\ &\quad + (P_{\text{dry}} \text{ and } P_{\text{pp}})], \text{ and} \\ \text{New IMI} &= (N_{\text{dry}} \text{ and } P_{\text{pp}}) / [(N_{\text{dry}} \text{ and } N_{\text{pp}}) \\ &\quad + (N_{\text{dry}} \text{ and } P_{\text{pp}})], \end{aligned}$$

where  $N_{\text{dry}}$  and  $P_{\text{dry}}$  represent infectious status at dry off and  $N_{\text{pp}}$  and  $P_{\text{pp}}$  represent infectious status postpartum. If an udder quarter was positive for 1 bacterial species at dry off and for another bacterial species postcalving, the elimination rate was calculated for the bacterial species occurring at dry off and a new IMI rate for the bacterial species occurring postcalving.

### Analytical Determination of Inflammatory Response in the Udder Quarter Milk Samples

Milk Hp concentrations (mg/L) were determined by a method based on the ability of Hp to bind to hemoglobin adapted to be used for milk, as described by Kalmus et al. (2013). Optical densities of the formed complex were measured at 450 nm using a spectrophotometer. Lyophilized bovine acute phase serum was used as a standard, and calibration was carried out according to the European Union concerted action on the standardization of animal acute phase proteins number QLK5-CT-1999-0153 (Skinner, 2001). The working range of the assay was 60 to 1,900 mg/L. The inter- and intra-assay coefficient of variation values for Hp analysis were <12 and <9%, respectively.

### Statistical Analyses

The qPCR test results were dichotomized for each bacterium as either presence or absence of udder pathogen by using the cutoff values ( $\leq 37.0$ ) set by the manufacturer. Cow parity was categorized into 1, 2, 3, and  $\geq 4$  lactations and DIM into  $\leq 300$  and  $\geq 301$ . According to visual evaluation, the presence of blood

in milk samples was dichotomized as either presence or absence (yes = 1, no = 0).

Two models were used separately for investigating associations between milk Hp concentration and udder pathogens at dry off and after calving. To achieve a normal distribution of the outcome variable, inverse square root (1/square root of Hp) transformation was used. According to the causal diagram, the presence of blood in the milk sample, cow parity, DIM, and the length of the dry period were possible confounders in these models. A mixed tobit regression model (Figure 1) was used for estimating the associations between the milk Hp concentration and the presence of udder pathogens in udder quarter milk samples at dry off, as, at dry off, 17.8% of milk samples were under the detection limit for Hp concentrations, which would violate the regression model's assumptions. In the tobit regression, all cases above (or below) a specific threshold value were censored, although these cases remained in the analysis. A mixed linear regression model (Figure 2) was used to estimate the associations between milk Hp level and the presence of udder pathogens in udder quarter milk samples after calving. The cow was included as a random factor to both models.

In both models, interaction terms were tested for significance to determine whether the combined effect of 2 udder pathogens differed from the sum of the individual effects of the pathogens tested.  $P$ -values  $\leq 0.05$  were considered statistically significant. Assumptions of the equal variance of the outcome in all levels of predictor variables and normal distribution of the residuals were checked graphically. Stata IC 10 (StataCorp, College Station, TX) software was used for statistical analyses.

## RESULTS

### Identification of Mastitis Pathogens

In total, 1,001 udder quarter milk samples were collected at dry off ( $n = 510$ ) and after calving ( $n = 491$ ) from 133 dairy cows. At dry off, 191 (37.5%) udder quarters out of 510 were positive for 1 or more of the detected udder pathogens. The most prevalent udder pathogen was *Strep. agalactiae* ( $n = 132$ ; 25.9%), followed by *Staph. aureus* ( $n = 63$ ; 12.4%) and *M. bovis* ( $n = 15$ ; 2.9%).

After calving, 57 (11.6%) of 491 udder quarter milk samples were positive for detected udder pathogens. *Mycoplasma bovis* and *Staph. aureus* were both identified in 17 (3.5%) udder quarter milk samples. *Streptococcus agalactiae* and *Strep. uberis* were identified in 14 (2.9%) and 15 (3.1%) udder quarter milk samples, respectively.

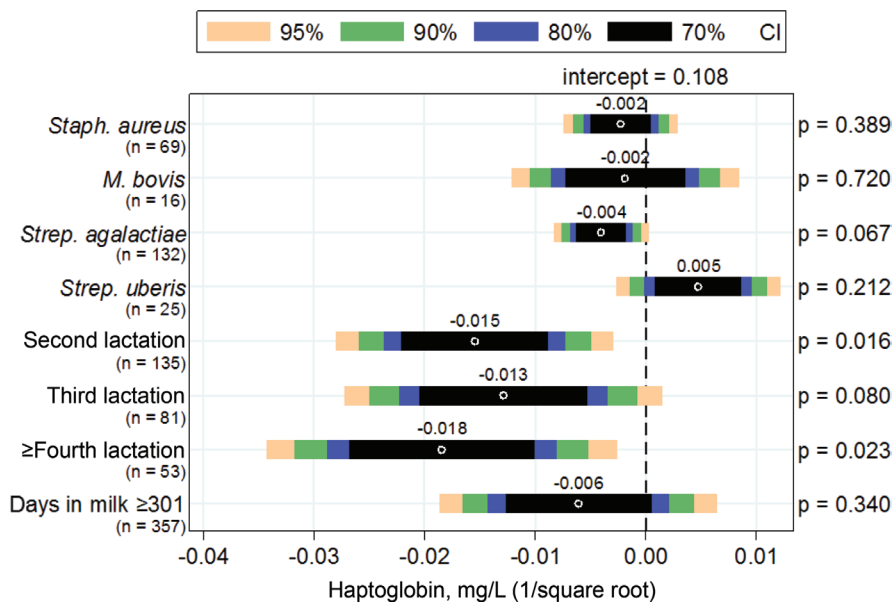


Figure 1. Results of mixed tobit regression model (86 right-censored samples) of associations between udder quarter milk samples ( $n = 510$ ) haptoglobin (Hp) concentration (mg/L) after 1/square root transformation (because of inverse transformation negative coefficient means positive effect) and udder pathogens at the dry off. Model confidence intervals are presented as horizontal bars. Point estimates for variables are shown on top of the bars. Cow lactation (compared with first-lactation dairy cows;  $n = 217$ ) and DIM were retained in the model as confounders. Color version available online.

#### Elimination of Udder Pathogens During the Dry Period

Among detected udder pathogens, *Strep. uberis* had the highest elimination rate (100.0%). Elimination rates for *M. bovis*, *Staph. aureus*, and *Strep. agalactiae* were 86.7, 93.6, and 96.2%, respectively (Table 1).

#### Inflammatory Response in the Udder Quarters Before and After Dry Period

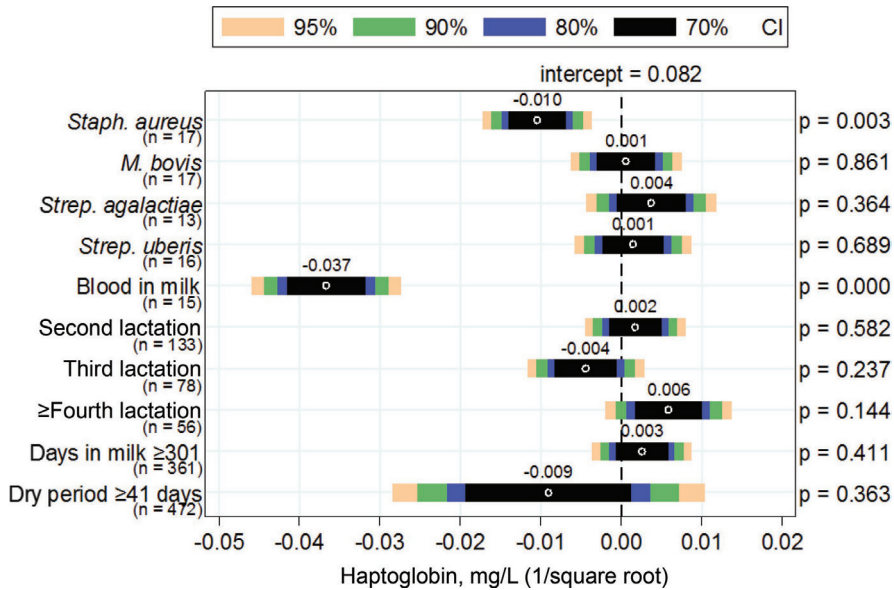
The milk Hp concentration was below the working range ( $\leq 60$  mg/L) in 91 (17.8%) out of 510 udder quarter milk samples at dry off. After calving, the milk Hp concentration was below the working range in 1.8% ( $n = 9$ ) of 491 udder quarter milk samples. In 10 (2%) postpartum and 4 (0.8%) dry off udder quarter milk samples with blood, the milk Hp concentration ranged between 181 and 4,096 mg/L.

The milk Hp concentration was higher in milk samples positive for *Strep. agalactiae* at dry off compared with milk samples negative for *Strep. agalactiae* (Figure 1). After calving, the milk Hp concentration was significantly higher in milk samples with blood and in milk samples positive for *Staph. aureus* compared with milk samples without blood and milk samples negative for *Staph. aureus* (Figure 2).

#### DISCUSSION

##### Elimination of Udder Pathogens During the Dry Period

The aim of our study was to evaluate the elimination of 4 udder pathogens from infected udder quarters during the dry period in cows treated with cloxacillin at dry off. Because only 1 herd was used for our study, the results are not directly comparable to other farms



**Figure 2.** Results of mixed linear regression model of associations between udder quarter milk samples (n = 479) haptoglobin (Hp) concentration (mg/L) after 1/square root transformation (because of inverse transformation negative coefficient means positive effect) and udder pathogens after calving. Model confidence intervals are presented as horizontal bars. Point estimates for variables are shown on top of the bars. Cow lactation (compared with first-lactation dairy cows; n = 216), DIM, and the length of dry period were retained in the model as confounders. Color version available online.

with different management practices and use of dry cow antibiotic therapy.

We identified a high elimination rate (86.7%) for *M. bovis* during the dry period. *Mycoplasma bovis* is resistant to many antibiotics, including cloxacillin (Rosenbusch et al., 2005), and *M. bovis* IMI is usually

**Table 1.** Elimination and new IMI rate in udder quarter milk samples during the dry period (n = 513)

Item	Elimination rate, <sup>1</sup> n (%)	New IMI rate, <sup>2</sup> n (%)
<i>Mycoplasma bovis</i>	13/15 (86.7)	15/498 (3.0)
<i>Staphylococcus aureus</i>	59/63 (93.6)	13/450 (2.9)
<i>Streptococcus agalactiae</i>	127/132 (96.2)	9/381 (2.4)
<i>Streptococcus uberis</i>	23/23 (100.0)	15/490 (3.1)

<sup>1</sup>Number of negative udder quarter milk samples postcalving, positive at dry off/number of positive udder quarter milk samples at dry off.

<sup>2</sup>Number of positive udder quarter milk samples postcalving, negative at dry off/number of negative udder quarter milk samples at dry off.

considered untreatable with dry cow therapy (Ruegg and Erskine, 2015). Hence, the elimination of *M. bovis* during the dry period is probably caused by other factors, such as altered cow immunity or unsuitable conditions for mycoplasma cells in dry mammary glands. Because of the small sample size, our results are only indicative, and further studies should investigate the elimination of *M. bovis* during the dry period in a larger study population.

Additionally, we identified a high elimination rate for *Strep. agalactiae* and *Strep. uberis* during the dry period, which is in line with previous studies (Dufour and Dohoo, 2013; Bradley et al., 2015). The elimination rate for *Staph. aureus* was also high in our study. The recovery from *Staph. aureus* IMI during the dry period is described to be more difficult compared with *Strep. agalactiae* IMI and to depend on the strain of *Staph. aureus*, with some strains of *Staph. aureus* being naturally more susceptible for elimination by antibiot-

ics (Dingwell et al., 2006). We did not use genotyping methods for the characterization of the detected *Staph. aureus*, so we cannot draw any conclusions about the possible strain variation as a cause for the high elimination rate of *Staph. aureus*. An alternative explanation for the high elimination rate of *Staph. aureus* could be the parity of cows. A higher probability for elimination of *Staph. aureus* IMI during the dry period is described in cows with lower parity; that is with younger cows (Dingwell et al., 2006). In our study, most of the cows were in their first or second lactation, which may explain the better elimination rate of *Staph. aureus* during the dry period.

A high sensitivity of the PCR method to detect udder pathogens from milk samples was reported in Koskinen et al. (2010). To our knowledge, ours is the first study in which commercial qPCR analysis was used to detect mastitis pathogens in quarter milk samples at dry off and after calving. However, we collected cow udder quarter milk samples only once before and after the dry period, which may have increased the probability of false-negative and false-positive test results. As cows do not excrete a steady number of bacteria with milk all the time, the amount of bacterial DNA in negative udder quarter milk samples may have been below the qPCR detection limit, giving false-negative results. On the other hand, a sensitive qPCR detects the bacterial DNA also from nonviable bacteria (Nyman et al., 2016; Parker et al., 2018). Hence, in our study some qPCR-positive udder quarter milk samples may have been falsely positive due to bacterial DNA from bacteria already killed by the cows' immune system. Repeated udder quarter milk sampling could have reduced the probability of false-negative and false-positive qPCR test results.

Cow udder quarter milk samples were analyzed with qPCR test kit detecting DNA of *Strep. agalactiae*, *Staph. aureus*, *Strep. uberis*, and *M. bovis*; therefore, we cannot draw any conclusions about the elimination of other udder pathogens. Pathogen-negative udder quarter milk samples in our study may have been positive for other udder pathogens, such as CNS, which are not detected by qPCR test kit Mastitis 4B. Additionally, this test kit detects udder pathogens at the species level; however, different strains of bacteria may circulate in the farm causing IMI. As we did not do any strain analysis, we cannot make any judgements about the variation in bacterial strains.

#### **Inflammatory Response in the Infected Udder Quarters Before and After Dry Period**

We evaluated milk Hp concentration in udder quarter milk samples at dry off and at first milking

postpartum to find associations between the milk Hp level and the presence of detected udder pathogens in udder quarter milk samples. We found elevated milk Hp concentrations in milk samples positive for *Strep. agalactiae* at dry off and in milk samples positive for *Staph. aureus* after calving compared with udder quarter milk samples negative for these pathogens. Both *Strep. agalactiae* and *Staph. aureus* usually cause chronic subclinical mastitis (Keefe, 2012). Eckersall et al. (2006) found that experimentally induced subclinical *Staph. aureus* IMI increased milk Hp concentration. In our study, elevated milk Hp concentration in udder quarter milk samples positive for *Strep. agalactiae* at dry off and for *Staph. aureus* postpartum may indicate a present or recent subclinical IMI caused by these udder pathogens. Milk Hp may be affected by multiple udder pathogens, and the number of bacteria detected in our study was limited to 4 main gram-positive udder pathogens. Therefore, the milk Hp concentration in udder quarter milk samples may have been affected by udder pathogens not detected in the qPCR test kit used in our study. Further studies with larger sample size are needed to evaluate the association between subclinical udder inflammation and the milk Hp concentration, and the results of our study should be considered as indicative.

We did not find a significant correlation between the milk Hp concentration and the presence of *M. bovis* in udder quarter milk samples at dry off or after calving. To our knowledge, no published data exists about the presence of *M. bovis* in udder quarters and local inflammatory response in mammary gland measured via milk Hp concentration. Therefore, further studies are needed to understand the factors affecting milk Hp concentration in *M. bovis*-positive udder quarters.

## **CONCLUSIONS**

The elimination of *Strep. agalactiae*, *Staph. aureus* and *Strep. uberis* during the dry period was high. The elimination of *M. bovis* during the dry period was lower compared with the elimination of *Strep. agalactiae*, *Staph. aureus*, or *Strep. uberis*. Results of this study indicate that spontaneous elimination of *M. bovis* probably occurs during the dry period. However, further studies should investigate the elimination rate of *M. bovis* during the dry period in a larger population. Higher milk Hp concentrations at dry off were identified in *Strep. agalactiae*-positive udder quarter milk samples compared with pathogen-negative udder quarter milk samples; the same association was found for *Staph. aureus* postpartum. We can conclude that the milk Hp concentration can be used as an indicator of the pres-

ence of udder inflammation at dry off and after calving together with bacteriological results.

#### ACKNOWLEDGMENTS

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## CURRICULUM VITAE

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2018–2020 Research project founded by the Estonian Agricultural Registers and Information Board “Development of biosensor for early detection of mastitis” (T180198VLKM).

## List of all publications

### 1.1 Scholarly articles indexed by Web of Science Science Citation Index Expanded, Social Sciences Citation Index, Arts & Humanities Citation Index and/or indexed by Scopus

Timonen, Anri A. E.; Autio, Tiina; Pohjanvirta, Tarja; Häkkinen, Liidia; Katholm, Jørgen; Petersen, Anders; Mõtus, Kerli; Kalmus, Piret (2020). Dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds. *Veterinary Microbiology*, 242, 108608.10.1016/j.vetmic.2020.108608.

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### 3.5. Articles/presentations published in local conference proceedings

Timonen, Anri Aino Elisa; Katholm, Jørgen; Petersen, Anders; Mõtus, Kerli; Kalmus, Piret (2017). *Mycoplasma bovis*'e põhjustatud udaranakkuse karjasisene levimus, mõju lehma udaratervisele, piimatoodangule ja koostisele. Marko Kass (\_EditorsAbbr). Conference “Terve loom ja tervislik toit 2017” proceedings: Terve loom ja tervislik toit, Tartu, 1-2. märts 2017 (89–98). Tartu: Eesti Maaülikool.

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### 6.3. Popular science articles

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Timonen, Anri Aino Elisa; Kalmus, Piret; Tummeleht, Lea (2017). Veiste udarapõletiku diagnoosimine PCR-meetodil. Eesti Loomaarstlik Ringvaade, 4, 6–9.

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## Kõik ilmunud artiklid

1.1. Teadusartiklid, mis on kajastatud Web of Science andmebaasides Science Citation Index Expanded, Social Sciences Citation Index, Arts & Humanities Citation Index ja/või andmebaasis Scopus

Timonen, Anri A. E.; Autio, Tiina; Pohjanvirta, Tarja; Häkkinen, Liidia; Katholm, Jørgen; Petersen, Anders; Mõtus, Kerli; Kalmus, Piret (2020). Dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds. *Veterinary Microbiology*, 242, 108608.10.1016/j.vetmic.2020.108608.

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3.5. Artiklid/ettekanded, mis on avaldatud kohalikes konverentsikogumikes

Timonen, Anri Aino Elisa; Katholm, Jørgen; Petersen, Anders; Mõtus, Kerli; Kalmus, Piret (2017). *Mycoplasma bovis*'e põhjustatud udaranakkuse karjasisene levimus, mõju lehma udaratervisele, piimatoodangule ja koostisele. Marko Kass (\_EditorsAbbr). Konverentsi ”Terve loom ja tervislik toit 2017” artiklite kogumik: Terve loom ja tervislik toit, Tartu, 1-2. märts 2017 (89–98). Tartu: Eesti Maaülikool.

5.2. Publitseeritud konverentsiteesid

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Timonen, Anri Aino Elisa; Kalmus, Piret; Tummeleht, Lea (2017). Veiste udarapõletiku diagnoosimine PCR-meetodil. Eesti Loomaarstlik Ringvaade, 4, 6–9.

# VIIS VIIMAST KAITSMIST

## TARMO NIINE

IMPACT OF GASTROINTESTINAL PROTOZOAN INFECTIONS ON THE ACUTE  
PHASE RESPONSE IN NEONATAL RUMINANTS  
SEEDEKULGLAT TÕVESTAVATE ALGLOOMADE MÕJU MÄLETSEJALISTE ÄGEDA  
JÄRGU VASTUSELE NEONATAALPERIOODIL

Professor **Toomas Orro**, doktor **Brian Lassen**

21. november 2019

## IVAR OJASTE

BREEDING AND MIGRATION ECOLOGY OF EURASIAN CRANE (*GRUS GRUS*)  
SOOKURE (*GRUS GRUS* L.) PESITSUS- JA RÄNDEÖKOLOOGIA

Professor **Kalev Sepp**, vanemteadur **Aivar Leito**†, vanemteadur **Ülo Väli**

1. detsember 2019

## BIRGIT AASMÄE

ANTIMICROBIAL RESISTANCE OF ESCHERICHIA COLI AND ENTEROCOCCI  
ISOLATED FROM SWINE, CATTLE AND DOGS AND MASTITIS PATHOGENS  
ISOLATED IN ESTONIA IN 2006–2015.

EESTIS AASTATEL 2006–2015 SIGADELT, VEISTELT JA KOERTEL  
ISOLEERITUD ESCHERICHIA COLI JA ENTEROCOCCUS'E PEREKONNA  
MIKROOBIDE NING LEHMADELT ISOLEERITUD MASTIIDIPATOGEENIDE  
ANTIBIOOTIKUMIRESISTENTSUS.

Dotsent **Piret Kalmus** ja professor **Toomas Orro**

13. detsember 2019

## KAIA KASK

THE EFFECTS OF HEAT STRESS SEVERITY ON PHOTOSYNTHESIS AND  
VOLATILE ORGANIC COMPOUND EMISSIONS IN BLACK MUSTARD AND  
TOBACCO.

KUUMASTRESSI MÕJU MUSTA KAPSASROHU (*BRASSICA NIGRA* L.)  
JA VÄÄRISTUBAKA (*NICOTIANA TABACUM* L.) FOTOSÜNTEESILE JA  
LENDUVÜHENDITE EMISSIOONIDELE.

Professor **Ülo Niinemets**, vanemteadur **Astrid Kännaste**

5. märts 2020

## MÄRT REINVEE

APPLICABILITY OF LOW-COST ELECTROMYOGRAPHS IN ERGONOMIC  
ASSESSMENT

MADALA MAKSUMUSEGA ELEKTROMÜOGRAAFIDE RAKENDATAVUS  
ERGONOOMIKALISES HINDAMISES

Emer. dots. **Jaak Jaaniste**, prof. **Mati Pääsuke**

13. märts 2020

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