Mycoplasma bovis:

Sources of infection, prevalence and risk factors

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AI	Artificial insemination
BAL	Broncho alveolar lavage
BB	Belgian blue
BRD	Bovine respiratory disease
BTM	Bulk tank milk
CFU	Colony forming units
CI	Confidence interval
CT	Computed tomography
Ct-value	Cycle threshold value
DNS	Deep nasal swab
ELISA	Enzyme-linked immunosorbent assay
FPT	Failure of passive transfer
HF	Holstein Friesian
HR	Hazard ratio
IS typing	Insertion sequence typing
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable-number tandem-repeat analysis
OR	Odds ratio
ODC%	Optical density coefficient
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PPLO	Pleuropneumonia like organism
RAPD	Random amplified polymorphic DNA
SD	Standard deviation
SSC	Somatic cell count
Vsp	Variable surface lipoproteins

Mycoplasma bovis can be the bane of a farmer's existence. As one of the most important members of the bovine *Mollicutes*, it causes a multitude of diseases, most notable of which are mastitis, pneumonia and arthritis in adult cows and pneumonia, arthritis and otitis in calves. *M. bovis* infections have a tremendous adverse impact on the economic output of the farm, its antimicrobial use and the welfare of the cattle affected. Although the pathogen is present in most countries worldwide, prevalence differs a lot. Introduction into farms is generally caused by the purchase of a (sub)clinically infected animal, but a multitude of other possible ways of introduction have been suggested in the past, which need to be evaluated in the effort to keep herds disease-free.

Due to the chronic nature of mycoplasmal disease and the presence of subclinical shedders, detecting the infection can be difficult. Treatment is often disappointing as well, due to *M. bovis*' innate resistance to multiple antimicrobials and chronicity linked to several virulence factors. Decreasing susceptibility to antimicrobial agents has been reported as well, with country specific differences. Treatment failure happens so often, that for certain *M. bovis* caused diseases such as mastitis the current advice is to cull all affected animals.

Since effective treatment is difficult, focus should be on disease prevention. Unfortunately, the design of a *M. bovis* vaccine appears to be difficult, hence its absence in Europe as a tool to control *M. bovis* infections. Through epidemiological research, certain risk factors such as purchase of replacement animals and insufficient milking hygiene have been identified. However, a lot more research is needed to fully understand the way *M. bovis* can enter herds and migrate throughout the herd, before effective control and prevention programs can be developed.

This thesis aimed to fill in some of the gaps still present in our epidemiological knowledge of *M. bovis*, to aid in the development of new, better preventive measures to contain the ongoing spread of *M. bovis*.

CHAPTER 1

GENERAL INTRODUCTION

Mycoplasma bovis: an overview

1. ETIOLOGY AND CHARACTERISTICS

Mycoplasma bovis, previously called *Mycoplasma agalactiae var. bovis* or *Mycoplasma bovimastitidis*, was probably first described in 1954 as a case of bovine pneumonia (Carter, 1954; Jasper et al., 1974b; Askaa and Erno, 1976; Pfützner and Sachse, 1996). The first isolation out of bovine mastitis followed 8 years later, in 1962 (Hale et al., 1962). In the following decades, *M. bovis* has spread throughout the world (ter Laak et al., 1992; Spergser et al., 2013; Aebi et al., 2015). Because of the increasing movement of cattle across countries, only a few countries managed to steer clear of the bacterium (Reeve-Johnson, 1999). In July 2017, *M. bovis* was found in New Zealand as well, one of the last remaining *M. bovis* free countries until then (Ministry for Primary Industries, 2017).

The economic impact of *M. bovis* caused diseases is hard to encompass. Due to the chronicity of infections with this bacterium the total loss of weight gain, feed conversion, therapy costs and man hours is often unknown (Caswell and Archambault, 2008). Purely based on loss of carcass value and decreased weight gain, the cost for the US beef industry alone has been estimated to lay around 32 million dollars a year in 1999, which would be around 48 million dollars in 2018 accounting for inflation (Rosengarten and Citti, 1999). The decreased weight gain can be up to 800g for each week of pneumonia in the first three months of age (Reeve-Johnson, 1999). When looking at *M. bovis* mastitis related losses in the US dairy industry, this cost goes up to 108 million dollars a year (136 million when accounting for inflation) (Rosengarten and Citti, 1999). In 2003, the yearly economic losses due to Bovine Respiratory Disease (BRD) and related illnesses across Europe were estimated to be 576 million euros (733 million euros when accounting for inflation), of which 25% - 33% could be attributed to *M. bovis* (Nicholas and Ayling, 2003). Of course, next to the economic costs, the welfare impact of the disease should not be underestimated, as *M. bovis* is often the cause of chronic illness with less than optimal response to treatment (Maunsell et al., 2011).

M. bovis is part of the genus *Mycoplasma* within the *Mycoplasmacetae*, which is a family within the class Mollicutes. Mollicutes (literally translated as "soft skinned") are a specific class of bacteria, unique because of their lack of a cell wall. Originally stemming from a gram-positive lineage, they are among the smallest self-replicating organisms (200-300 nm), with a very small genome as well (Razin, 1992; Caswell and Archambault, 2008).

Mycoplasmataceae are mainly parasitic in nature with, so far, around 180 known species infecting mammals, reptiles, fish, plants and arthropods (Razin et al., 1998). The different species generally are rather strict tissue- and host specific, but can on occasion cross to other tissues or hosts (Madoff et al., 1979; Razin et al., 1998; Pitcher and Nicholas, 2005). Next to *M. bovis*, several other species of *Mycoplasma* are known cattle pathogens. *Mycoplasma mycoides subsp. mycoides* is the most pathogenic among them, as the cause of contagious bovine pleuropneumonia, the only bacterial disease on the former list A diseases of the Office International des Epizooties (Nicholas and Ayling, 2003). Next to *Mycoplasma*, there is one other genus of *Mycoplasmataceae* of importance for cattle, namely *Ureaplasma*, and one other family of importance in the Mollicutes class, the *Acholeplasmaceae* (Erno, 1987). An overview of the *Mycoplasma, Ureaplasma* and *Acholeplasma* species currently assumed to cause disease in cattle is shown in Table 1.

Geographical distribution of certain *Mycoplasma* species is possible: in the United States, only half of the *Mycoplasma* mastitis cases are caused by *M. bovis*, the remainder being caused by *M. californicum, M. bovigenitalium, M. alkalescens* and *M. canadense*. In Europe however, almost all reports list *M. bovis* as the main cause of *Mycoplasma* mastitis, though one of the most commonly used PCR mastitis tests (PathoProof™, Thermo Fisher Scientific, MA, USA) only tests for this species specifically (other *Mycoplasma* are grouped), possibly resulting in the under diagnosis of other *Mycoplasma* species in this part of the world (Fox, 2012; Nicholas et al., 2016). Even though *M. bovis* is primarily a cause of disease in cattle, it is also known to cause disease in other hosts on occasion, and could potentially spread from these hosts to other susceptible cattle again (Pfützner and Sachse, 1996). In buffaloes and bison, *M. bovis* is especially feared because of the high mortality associated with herd outbreaks (Bras et al., 2016; Calcutt et al., 2018). In Austria, *M. bovis* was isolated from pigs that were housed on the same pasture and stables as a cattle herd which was experiencing a dramatic primary outbreak of *M. bovis* associated diseases. The pigs showed clinical respiratory disease, with conjunctivitis, nasal discharge and coughing (Spergser et al., 2013). Reports of isolation of *M. bovis* out of (mainly) respiratory infections are available for sheep, goats, deer and poultry as well (Damassa et al., 1992; Dyer et al., 2004; Ongor et al., 2008; Kumar et al., 2012). *M. bovis* can act as a zoonosis, primarily in immunosuppressive patients, and has on occasion caused systemic illness (Madoff et al., 1979; Pitcher and Nicholas, 2005).

Table 1: *Mycoplasma (M.) , Ureaplasma (U.)* and *Acholeplasma (A.)* species with a predilection for cattle

Name	Main clinical signs	Present in Belgium	Source
A. axanthum	Mastitis, possible pneumonia	?	Pfützner et al., 1979, Reeve-Johnson, 1999
A. laidlawii	Mastitis, reproductive tract lesions	?	Pfützner et al., 1979, Doig, 1981, Kirkbride, 1987
A. modicum	Possible pneumonia	?	Reeve-Johnson, 1999
M. alkalescens	Mastitis, arthritis, pneumonia	Yes	Bennett and Jasper, 1978, Jasper, 1980, Thomas et al., 2003, Kokotovic et al., 2007
M. alvi	Isolated out of intestinal and urogenital tract, importance unknown	?	Gourlay et al., 1977, Nicholas et al., 2008
M. arginini	Mastitis, pneumonia	Yes	Muenster et al., 1979, González and Wilson, 2003
M. bovigenitalium	Genital infections both genders, mastitis, pneumonia	?	Gourlay et al., 1979, Jasper, 1980, Fox, 2012
M. bovirhinis	Often found, relevance questionable, possible involvement in pneumonia and mastitis	Yes	Muenster et al., 1979, Thomas et al., 2003, Fox et al., 2005
M. bovis	Pneumonia, mastitis, otitis, arthritis, meningitis, infectious keratoconjunctivitis, oophoritis, salpingitis, abscesses, abortion, polyserositis, infertility, pericarditis	Yes	Hirth et al., 1966, Kinde et al., 1993, Adegboye et al., 1996, Pfützner and Sachse, 1996, Thomas et al., 2003, Gagea et al., 2006, Nicholas et al., 2008
M. bovoculi	Infectious keratoconjunctivitis, mastitis	?	Fox et al., 2005, Schnee et al., 2015
M. californicum	Mastitis, pneumonia, arthritis	?	Jasper, 1980, Hewicker-Trautwein et al., 2002, Fox, 2012
M. canadense	Mastitis	?	González and Wilson, 2003
M. canis	Pneumonia, mastitis, part of the microflora of the bovine respiratory tract	Yes	ter Laak et al., 1993, Thomas et al., 2003, Fox et al., 2005, Nicholas et al., 2008
M. dispar	Pneumonia, immunosuppression, mastitis	Yes	Gourlay et al., 1979, Howard et al., 1987, Thomas et al., 2003, Fox et al., 2005, Nicholas et al., 2008
Candidatus M. haemobos *(M. haemobovis)*	Infectious anemia (Haemoplasma), hock swelling, fever, transplacental infection with possible abortion	?	Ayling et al., 2012, Girotto-Soares et al., 2016
M. mycoides subsp. mycoides	Contagious bovine pleuropneumonia: Pneumonia, fever, arthritis, death	No	Provost et al., 1987, Nicholas et al., 2008
M. leachii sp. nov. (Leach's M. species group 7)	Mastitis, arthritis, abortion, pneumonia	?	Hum et al., 2000, Chang et al., 2011
M. verecundum	Conjunctivitis	?	Gourlay et al., 1974
M. wenyonii	Haemoplasma; edema, fever	Yes	Montes et al., 1994, Strugnell and McAuliffe, 2012, DGZ, 2015
U. diversum	Genital infections	Yes	Kirkbride, 1987, Thomas et al., 2003

2. PATHOGENESIS

Mycoplasma bovis has, just like many other *Mycoplasma* species, a tight association with the host cells (Caswell et al., 2010). Probably as a consequence of the small genome of said *Mycoplasma,* it has caused them to depend on the nutrition and growth of host cells to ensure their survival (Maunsell and Donovan, 2009).

Surface adhesion is, in part, mediated by so called variable surface lipoproteins (Vsp) (Sachse et al., 2000; Caswell et al., 2010). Laying on the surface of *M. bovis* plasma membrane, in total, 13 different Vsps have been identified, all of them having antigenic properties stimulating the hosts immune response (Lysnyansky et al., 2001; Perez-Casal et al., 2017). Thanks to genes that allow variable (uncoordinated) expression of these Vsps (ON or OFF), *M. bovis* is capable of rapid variability in the antigenic expression of their outer cell membrane proteins, effectively evading the hosts antibodies (Razin et al., 1998; González and Wilson, 2003). Antibody expression to a specific Vsp can even result in the selection of variant mycoplasmas expressing OFF in that Vsp (González and Wilson, 2003). Furthermore, due to different expression of proteins, Vsps will have size variations as well, further resulting in variability of the antigenic expression (Lysnyansky et al., 2001). Different *M. bovis* strains can have different versions of the Vsp encoding gene complex, leading to a multitude of phenotypic variations (Razin et al., 1998; Perez-Casal et al., 2017). Next to the Vsps, also other membrane proteins have been found, providing even more surface membrane diversification (González and Wilson, 2003). The rapidly changing and diverse expression of these antigens on the plasma membrane is suspected to play a major role in the difficulty of producing large scale effective vaccines against *M. bovis* (Perez-Casal et al., 2017*)*.

Infection with *M. bovis* evokes a robust localized and systemic immune response (Caswell et al., 2010). In serum, an IgG and IgM reaction can be seen, whereas in nasal and lung fluids the reaction is mainly IgA based (Caswell et al., 2010). The IgG immune response seems to be mainly composed of IgG1, whereas IgG2 usually has the superior opsonin activity, possibly contributing to the chronic nature of *M. bovis* infections (Vanden Bush and Rosenbusch, 2003). Furthermore, the immune response is also skewed towards T helper 2 cells, with an inhibition of the Th1 response which would provide a superior immunity (Vanden Bush and Rosenbusch, 2003).

Long term survival of *M. bovis* in necrotic tissue has been described, in the presence of phagocytic cells such as macrophages and neutrophils (Kleinschmidt et al., 2013). This could be an indication that the chronic persistence of *M. bovis* is also due to a resistance to phagocytosis (Kleinschmidt et al., 2013). Next to this, *M. bovis* was shown to suppress lymphocyte proliferation and inhibit the oxidative burst of neutrophils, further weakening the immune response (Thomas et al., 1991; Srikumaran et al., 2007). *M. bovis* has been shown to reside intracellularly in peripheral blood mononuclear cells (B cells, T cells, monocytes and others) and erythrocytes, possibly using these circulating blood cells as a means of transport throughout the hosts body and immunoevasion (van der Merwe et al., 2010). *M. bovis* was able to invade and multiply in embryonic turbinate cells as well, further demonstrating the possibility of immunoevasion and avoidance of antimicrobials by residing intracellularly (Burki et al., 2015).

M. bovis is able to activate complement and increase vascular permeability by the production of inflammatory substances such as H_2O_2 and toxins (Geary et al., 1981; Khan et al., 2005; van der Merwe et al., 2010). H_2O_2 production can cause contact-cytotoxicity to epithelial cells in theory (Bürki et al., 2015), but a study by Schott et al. (2014) failed to detect a difference in H_2O_2 expression between caseonecrotic pneumonia, other types of pneumonia and non-pneumonic lungs affected by *M. bovis*. They did detect immunohistological markers for lipid peroxidation and tyrosine nitration, which can contribute to the caseonecrotic lesions typical for *M. bovis* pneumonia and -arthritis (Devi et al., 2014; Schott et al., 2014). It has been speculated that the aforementioned domination of the (less effective) Th2 response is the result of oxygen free radical production by *M. bovis* (Schott et al., 2014).

M. bovis has the capacity to produce a biofilm, making it possible for the bacterium to survive in the environment for longer, while withstanding the stress of heat or desiccation (McAuliffe et al., 2006). There is a large amount of strain variability, with some *M. bovis* strains barely being able to form a biofilm or adhere to a surface compared to others, seemingly correlated to the expression of certain Vsps (McAuliffe et al., 2006). It was shown possible for *M. bovis* to remain viable in the environment for months at low temperatures, and weeks at room temperature on a variety of substrates (table 2) (Pfützner, 1984). The formation of a biofilm does not significantly influence the minimum inhibitory concentration (MIC) of antimicrobials to *M. bovis* compared to planktonic

strains (McAuliffe et al., 2006). However, it can increase the amount of self-induced damage to host tissues, by attracting and activating phagocytes while being protected from phagocytosis, inducing the phagocytes to release more lysosomal enzymes, reactive oxygen and nitrogen (Bürki et al., 2015).
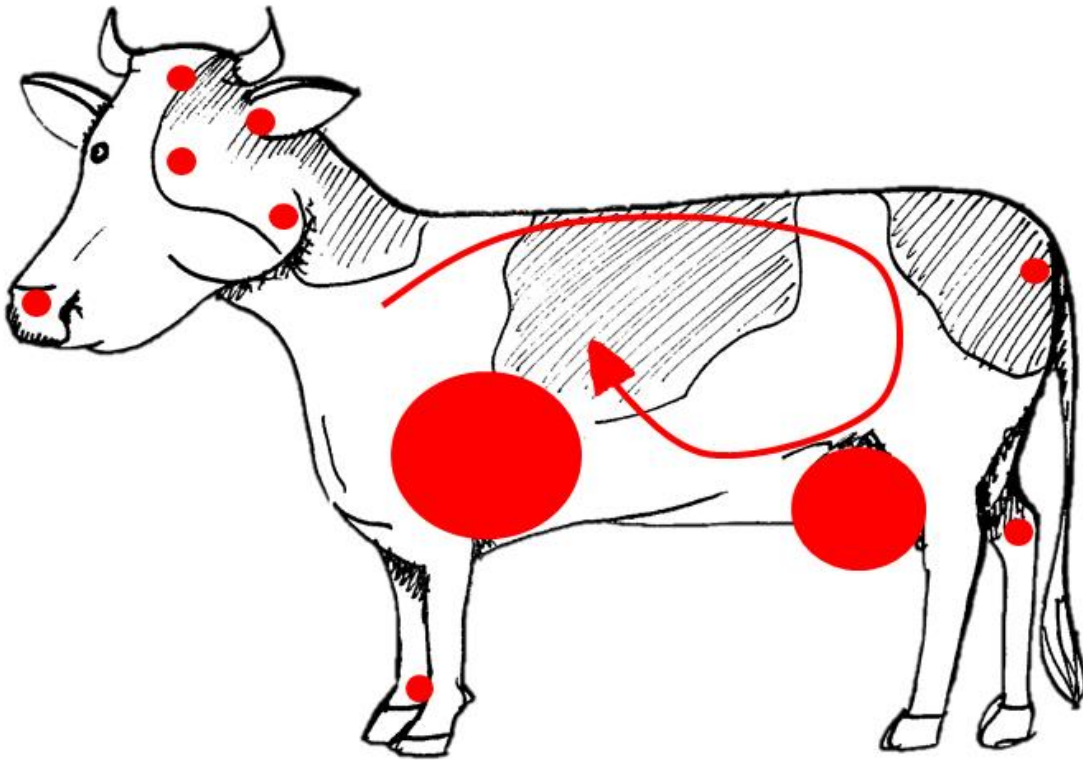
Figure 1: *Mycoplasma bovis* predilection sites

This figure portrays the most common predilection sites of *M. bovis*: nares, tonsillae, eyes, *Bulla tympanica*, meninges, lungs, udder, joints, genital area and hematological spread

3. PATHOLOGY AND DISEASE COURSE

M. bovis is chameleon-like, being able to change its behavior to cause a variety of clinical symptoms, grouped under the term "mycoplasmosis". Most commonly, mastitis and arthritis are observed in adult cattle, and pneumonia, arthritis and otitis in calves, combined with general signs of an infection, such as fever, depression and anorexia. Since *M. bovis* can spread hematogenously, symptoms in affected animals can vary and change in time (Rosengarten and Citti, 1999). The possibility of strains having a predilection for specific diseases has been suggested but more research is warranted on this topic (McAuliffe et al., 2004). An overview of the most common *M. bovis* predilection sites is shown in Figure 1.

3.1. MASTITIS

In adult cattle, *M. bovis* is mainly feared as a cause of mastitis (Figure 2). Highly contagious, it is a major cause of reduced welfare and milk production, especially in large dairy herds (Nicholas et al., 2016). In European countries, *Mycoplasma* mastitis is seen as a rising problem, though this could also be partially related to a lack of targeted searches in the past, as *M. bovis* is usually overgrown on standard bacterial culture (Nicholas et al., 2016).

Mycoplasma mastitis in a clinical stage is characterized by an altered milk consistency, a severe and sudden drop in milk production and a resistance to treatment (Jasper, 1982). Another typical trait of the infection is that multiple quarters can be affected, due to the capacity of the bacterium to spread hematogenously (Jasper, 1982;, Pfützner and Sachse, 1996; Rosengarten and Citti, 1999). Milk consistency can vary from watery to purulent, though a watery milk with a fibrinous sandy sediment is very typical for the infection (Jasper, 1982; Pfützner and Sachse, 1996). Incubation generally takes 2-10 days before mastitis is seen, during which shedding can already occur (Pfützner and Sachse, 1996; Al-Farha et al., 2017). Affected cattle can remain clinically normal as well, even when the udder has already been severely compromised, and can act as subclinical carriers of the bacterium for a longer period, shedding *M. bovis* intermittently (González and Wilson, 2003; Nicholas et al., 2016; Timonen et al., 2017). Subclinical carriers will often have an increased somatic cell count (SCC), a lower daily milk production (on average 3 l less) and a lower fat and urea content in milk (Al-Farha et al., 2017; Timonen et al., 2017). Shedding can persist throughout the lactation and even carry over into the next lactation, although some cows were reported to have eliminated the infection themselves (González and

Wilson, 2003). Cases of dry cows being affected by *M. bovis* mastitis without preexisting infection in a previous lactation have been reported (Pfützner and Sachse, 1996). Interestingly, *M. bovis* has been reported to cause mastitis in prepubertal heifers as well, resulting in nodules in the udder and one or multiple dry quarters in the first lactation (Fox et al., 2008). In these instances, hematogenous spread out of other sites of infection was assumed (Fox et al., 2008).

Histologically, acute *M. bovis* mastitis is characterized by degeneration of the epithelium of the alveoli combined with a leukocyte exudation (González and Wilson, 2003). Chronically, plasma cells will start to infiltrate the interalveolar space, combined with a progressive fibroplasia of the milk ducts and atrophy of the udder alveoli or even abscessation (González and Wilson, 2003). Antimicrobial therapy of *Mycoplasma* mastitis is often very disappointing, even when a systemic approach is combined with local treatment. As such, together with the inconsistent shedding and the variable duration of clinical symptoms, *M. bovis* mastitis is considered untreatable by a multitude of sources and affected cattle should be considered lifelong infected (Pfützner and Sachse, 1996; Nicholas et al., 2016; Timonen et al., 2017). As a consequence, most control programs recommend the culling of affected animals (Pfützner and Sachse, 1996; Nicholas et al., 2016). However, some researchers have reported success in treating *M. bovis* mastitis with intensive antimicrobial therapy (Byrne et al., 1998).

Even though *M. bovis* is often found together with other mastitis pathogens, no significant effect of *M. bovis* mastitis in coinfection with other bacteria was found in a study by Timonen et al. (2017). Al-Farha et al. (2017) did not see a difference in mastitic milk composition of *M. bovis* coinfection compared to conventional mastitis pathogens either.

3.2. PNEUMONIA

Respiratory disease due to *M. bovis* can develop in cattle of any life stage, even affecting neonatal calves as young as 5 days postpartum (Stipkovits et al., 2000). The disease is characterized by fever, anorexia, dyspnea, depression, coughing and rhinorrhea (Stipkovits et al., 2000). Most commonly, pneumonia due to *M. bovis* is seen in young calves between 2 to 6 weeks of age (Stipkovits et al., 2000; Maunsell and Donovan, 2009). Coinfection with Bovine Viral Diarrhea Virus can worsen the clinical signs (Shahriar et al., 2002; Gagea et al., 2006). As *M. bovis* can downregulate the immunological reaction, it can also act as a predilecting factor to facilitate infection with or worsen the symptoms of

other respiratory pathogens such as *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, Bovine Respiratory Syncytial Virus and others, which is why *M. bovis* is considered a part of the bovine respiratory disease complex (BRD) (Caswell and Archambault, 2008). *M. bovis* pneumonia is associated with four main kinds of lesion patterns: suppurative bronchopneumonia without necrosis, caseonecrotic bronchopneumonia, bronchopneumonia with coagulation necrosis foci and chronic bronchopneumonia with subsequent abscessation (Caswell and Archambault, 2008)(Figure 3).


Figure 2: Purulent mastitis in a Belgian Blue **Figure 3:** Caseonecrotic lesions and pneumonia of the lung (Source: Han Versnaeyen, DGZ) **Figure 4:** Fibrinous arthritis of the carpus due to *M. bovis* **Figure 5:** Calf with a typical head tilt due to *M. bovis*, combined with a failure to thrive compared to other calves of the same age group.

In a caseonecrotic bronchopneumonia, *M. bovis* can persist for a long time extracellularly in the necrotic foci, evading the immunity of the infected animal even when being surrounded by neutrophils and macrophages (Kleinschmidt et al., 2013). Lesions are often focused on the cranial and middle lung lobes, but can affect the rest of the lung too in severe cases (Caswell and Archambault, 2008). Caseonecrotic lesions composed of necrotic nodules (mm to cm in diameter) surrounded with consolidation of the lung on autopsy are seen as an indicator for the presence of *M. bovis* (Shahriar et al., 2002; Gagea et al., 2006). *M. bovis* pneumonia often turns chronic, with *M. bovis* persisting in the necrotic lesions as well.

3.3. ARTHRITIS

Although *M. bovis* associated arthritis is more commonly seen in calves, this disease form has been reported in adult cattle as well (Wilson et al., 2007; Maunsell and Donovan, 2009). Affected animals become acutely lame with swelling of joints and tendon sheaths and high fever (Stalheim and Page, 1975; Adegboye et al., 1996; Hewicker-Trautwein et al., 2002). Large rotator joints, such as for example shoulder, elbow, and knee are commonly affected (Maunsell and Donovan, 2009). When opened, a pyogranulomatous to serofibrinous synovitis, bursitis and/or tenosynovitis can be observed (Stipkovits et al., 1993; Adegboye et al., 1996) (Figure 4). The amount of joint fluid increases, and might be turbid and contain fibrin (Ryan et al., 1983). Cartilage will start eroding, and gets replaced by fibrous connective tissue (Ryan et al., 1983). In experimental conditions, ulceration of the distal planum of the knees was seen, with secondary rupture of the synovial sac (Stalheim and Page, 1975). The most commonly accepted pathway of infection of the joint is through hematogenous spread out of a primary site, usually the lungs (Thomas et al., 1986; Rosengarten and Citti, 1999). As such, animals affected with *M. bovis* induced arthritis will usually have lesions in other organs such as lungs or udder as well (Stalheim and Page, 1975; Adegboye et al., 1996; Gagea et al., 2006). Response to therapy is poor, affected animals are usually culled though arthrodesis can be an option if only one joint is affected (Van Huffel et al., 1989; Maunsell and Donovan, 2009). Persistence of *M. bovis* in the joint for up to 28 days has been described (Stalheim and Page, 1975).
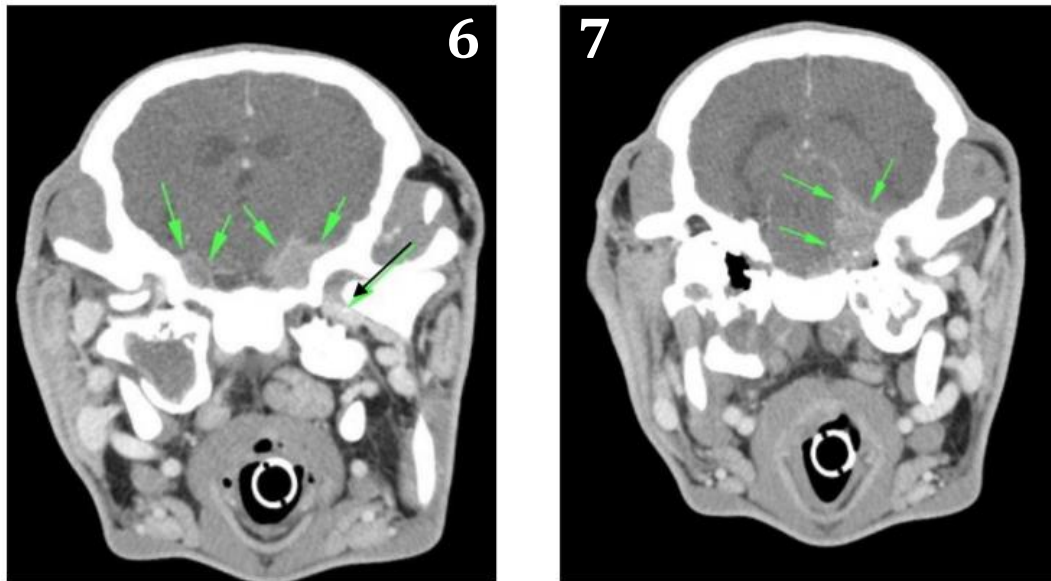
Figure 6 and 7: Contrast CT images showing a marked increase in contrast in the brain at the site of an otitis interna (green arrows). (Source: Linde Gille, 2014 master's thesis)

3.4. OTITIS

Mycoplasma bovis is a cause of otitis media in such a high number of cases, that the disease has been termed a "*Mycoplasma bovis* associated disease", just like pneumonia and arthritis, and can act as an indicator for *M. bovis* presence on a farm (Maunsell and Donovan, 2009). Other potential bacterial causes of both pneumonia and otitis are *H. somni, P. multocida* and *M. haemolytica* (Duarte and Hamdan, 2004). Calves affected with *M. bovis* otitis are generally lifeless, lay down more often compared to their non affected counterparts and usually portray a uni- or bilateral ear droop with a head tilt, fever and epiphora (Walz et al., 1997; Francoz et al., 2004; Foster et al., 2009) (Figure 5). Otitis media often causes chronic weightloss and wasting, and can result in otitis interna and meningitis with abscessation of the temporal bone if it goes untreated (Walz et al., 1997; Maeda et al., 2003)(Figure 6&7). On pathology, affected tympanic bullae are filled with a fibrinosupporative to caseous exudate (Walz et al., 1997). Cases are usually chronic and respond poorly to antimicrobial use (Maunsell and Donovan, 2009). The disease has been linked to the feeding of *M. bovis* infected waste milk or feed with subsequent colonisation of the tonsillae and eustachian tube, whereas transtracheal inoculation of *M. bovis* did not manage to induce otitis experimentally (Maeda et al., 2003; Foster et al., 2009; Maunsell et al., 2012).

3.5. REPRODUCTIVE DISEASES

Even though reproductive diseases caused by *M. bovis* were described as early as 1964, their importance in the *M. bovis* disease complex was seemingly disregarded later on (Doig, 1981; Maunsell et al., 2011). In recent years, the attention to these diseases is rising again, probably at least partially due to the unknown pathway of introduction of *M. bovis* in New Zealand and recent introductions via artificial insemination (AI) into *M. bovis* negative closed herds in Finland (Ministry for Primary Industries, 2017; Haapala et al., 2018).

Inoculation of *M. bovis* into the uterus of mature heifers leads to salpingitis, endometritis and salpingoperitonitis with ovarian adhesions (Hirth et al., 1966; Doig, 1981). Microscopically, chronic inflammation is present after inoculation of *M. bovis* in the uterine tract (Fabricant, 1973). Insemination with live *M. bovis* contaminated semen will lead to an increase in the number of inseminations required before conception (Hirth et al., 1966). *In vitro* as well, *M. bovis* infected semen will cause a lower number of developing embryos compared to control groups (Bielanski et al., 2000).

Even though the importance of *M. bovis* in the whole of bovine abortion cases is unknown, experimental inoculation of *M. bovis* into fetal membranes or intravenously can lead to abortion and *M. bovis* DNA has been detected in spontaneous abortion cases too (Stalheim and Proctor, 1976; Bocklisch et al., 1986; Kirkbride, 1987; Houlihan et al., 2007; Hermeyer et al., 2012).

In bulls, *M. bovis* can also be isolated from the genital tract, where it can induce seminal vesiculitis (Jasper et al., 1974a; LaFaunce and McEntee, 1982). Vesiculitis could not be reproduced when *M. bovis* was administered intravenously, only after local inoculation by LaFaunce and McEntee (1982). Even though natural cases of seminovesiculitis have been described, after preputial inoculation of *M. bovis*, Kreusel et al. (1989) did not manage to induce clinical disease except for mild local inflammation in some animals, indicating a possible strain dependence or interaction with other pathogens (Rosengarten and Citti, 1999). There seem to be differences in the distribution of *M. bovis'* presence in the seminal tract as well, as in Western Europe, Australia and America the bacterium was only seldom present in targeted searches, whereas in Hungary at one point 37% of all semen samples were positive for *M. bovis* (Kirkbride, 1987; Petit et al., 2008).

3.6. OTHER PRESENTATIONS

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In addition to the above mentioned *M. bovis* disease forms, several other presentations have been described to date. Since the bacterium can spread hematogenously, it is not rare to isolate it out of different organs. *M. bovis* can often be isolated out of conjunctival swabs in cattle affected with other symptoms of mycoplasmosis. When keratoconjunctivitis coincides with respiratory problems in a herd, *M. bovis* testing should be advised (Levisohn et al., 2004; Alberti et al., 2006). Kinde et al. (1993) described an outbreak of *M. bovis* infected subcutaneous decubital abscesses, where the area around the joints and brisket were affected without involvement of the joints. *M. bovis* meningitis can occur as a consequence of otitis interna, but has been described without the presence of an otitis interna as well (Stipkovits et al., 1993; Gosselin et al., 2012). In some cases, polyserositis and pericarditis was also present (Stipkovits et al., 1993).

Table 2: Sampling sites in live animals for different *M. bovis* presentations

Disease	Suggested sample	Test	Source
Mastitis	Milk	Culture, PCR, Ab ELISA	Parker et al., 2017b
Pneumonia	Bronchoalveolar lavage, transtracheal aspiration, DNS, conjunctival swab (might be more effective compared to DNS)	Culture, PCR	Sachse et al., 2010, Soehnlen et al., 2012
Arthritis	Synovial fluid	Culture, PCR	Maunsell et al, 2011
Otitis	Tonsil swabs, (DNS)	Culture, PCR	Maunsell et al, 2012
Keratoconjunctivitis	Conjunctival swab	Culture, PCR	Maunsell et al, 2011
Screening for carriers	DNS, vaginal swab, conjunctival swab, serum, milk, (BAL), semen	Culture, PCR, Ab ELISA	Soehnlen et al., 2012, Parker et al., 2017b, Hazelton et al., 2018
PCR: Polymerase Chain Reaction, Ab ELISA: Antibody Enzyme-Linked Immunosorbent Assay; DNS: Deep Nasopharyngeal Swab			

4. DIAGNOSIS

4.1. ANIMAL LEVEL

The appearance of typical clinical symptoms like otitis, arthritis or therapy resistant mastitis in multiple animals is usually the first sign of *M. bovis* introduction into a herd (Pfützner and Sachse, 1996). Early diagnosis at animal level is necessary, also when trying to remove carriers from a herd (Pfützner and Sachse, 1996). Sampling of the affected organ or one of the known carrying sites of *M. bovis* will give the highest sensitivity for detection. However, since *M. bovis* can be shed intermittently, a negative sample is no conclusive indicator that the tested animal is not a carrier of *M. bovis* (González and Wilson, 2003). When trying to diagnose *M. bovis* pneumonia, 94% of all calves with pneumonic lesions tested positive on nasal swabs as well, providing a more easily accessible sample method (Soehnlen et al., 2012). Next to the live bacterium or *M. bovis* DNA, antibodies can be detected in various substrates as well. Important to note is that very little shedding at other body sites could be shown in cows having had a clinical *M. bovis* mastitis recently (Hazelton et al., 2018).

4.1.1 CULTURE

The routine diagnosis of *M. bovis* associated disease in an animal is often still made by culturing samples taken from an affected organ or a common carrying site (Parker et al., 2018). As a consequence of the hematologic spread of the bacterium, the same *M. bovis* strain can be present at multiple organ sites (Pfützner and Sachse, 1996; Biddle et al., 2005). Due to their minimalistic nature, mycoplasmas cannot synthesize essential amino acids and need highly enriched growth media with a pH of 7.3-7.8 in order to grow successfully (Razin et al.,1998).

Culture is preferably done on specific pleuropneumonia-like (PPLO) agars or in liquid growth media with added selective antimicrobials, at 37°C with an increased moisture level and the addition of 5-10% CO_2 (Bushnell, 1984; Pfützner and Sachse, 1996; Fox et al., 2003). Culture has a high sensitivity and specificity (up to 10-10^2 CFU/ml when liquid samples are inoculated), but takes time. Due to the relatively slow growth of *M. bovis*, definite interpretation of the plates as negative can only be done after 7-10 days (Bushnell, 1984; Pfützner and Sachse, 1996). However, plates should be checked daily by use of a (stereo-) microscope for early growth or contamination. *M. bovis* colonies have a typical fried egg shape, formed because the central zone of the colony grows denser and

into the medium, whereas the surrounding zone only grows on the surface (González and Wilson, 2003). Culture can lack specificity to distinguish between commensal and pathogenic *Mycoplasma* species, as multiple species can grow on *Mycoplasma* media (Fox et al., 2005). Several agars are available which will give a characteristic reaction in the presence of *M. bovis*, such as agars with added Tween 80 (Devriese and Haesebrouck, 1991) and *M. bovis* specific diagnostic agars which will give a color change when *M. bovis* is present (Mycoplasma Experience Ltd, Surrey, UK).

For milk samples, 10μL of milk is usually inoculated to detect *Mycoplasma*. As such, the detection limit of this technique is 100 CFU/mL, which can be improved by centrifuging the sample before inoculation (Parker et al., 2018). While culture is an easily accessible technique, there are a lot of caveats. Due to the slow growing nature of *M. bovis*, it will often get overgrown by contaminants before diagnosis is made. As such, sampling and subsequent storage need to be performed as sanitary as possible, and samples should be stored cooled or frozen if culture cannot happen immediately (Parker et al., 2018). However, freezing milk samples containing *M. bovis* will cause at least a 1 log reduction, possibly resulting in a false negative culture afterwards, especially when the samples have a relatively low number of *M. bovis* such as in the case of bulk tank milk (BTM) samples (Boonyayatra et al., 2010). Animals need to be shedding to test positive, which means a single culture can be insufficient to identify carriers (Biddle et al., 2003; Hazelton et al., 2018). When animals have been treated with antimicrobials, *M. bovis* might not grow anymore *in vitro*, possibly resulting in false negative results (Caswell and Archambault, 2008).

4.1.2 ANTIBODY ELISA

Antibodies against *M. bovis* can be detected in individual serum and milk samples 1-2 weeks after the initial *M. bovis* exposure by use of an enzyme-linked immunosorbent assay (ELISA) (Nicholas et al., 2002). This technique can be used on BTM samples as well (Boothby et al., 1987; Nielsen et al., 2015). The primary advantage of this technique compared to culture and PCR is that bacteria do not need to be present at the time of sampling for the animal to test positive (Petersen et al., 2018a). Antibodies were shown to stay present for at least 6 months after the initial exposure in a vaccination experiment, although recent work has shown a rapid decline of antibodies in milk after initial disease onset, with a mean drop below the cutoff ODC% at 65 days past infection and the lower

95% CI only being above the ODC% cutoff for 10 days (between 7 and 17 days after disease onset) when using a BIOX K302 ELISA (BioX)(Nicholas et al., 2002; Petersen et al., 2018a). Furthermore, not all affected cattle will develop high antibody titers, possibly decreasing the sensitivity of these tests (Maunsell et al., 2011). BIOX ELISA results were shown to have no correspondence with PCR and culture results except for what can be explained by chance, possibly resulting in an increased detection rate when using a combination of tests together (Parker et al., 2017a; Parker et al., 2017b).

Although specific ELISA kits on milk are often used to screen for shedders, the sensitivity of these tests drops rapidly when only systemic infection is present without udder involvement (Petersen et al., 2018a). Antibodies also stay present longer in infected quarters compared to non-infected quarters in the same animal, an indication that the immune response is mainly a localized immunity (Byrne et al., 2000; Petersen et al., 2018a). In general, due to the large individual variation in antibody production, the reliability of ELISA tests is deemed questionable, generally unreliable for individual diagnosis and probably leading to an underdiagnosis of shedders (Pfützner and Sachse, 1996; Petersen et al., 2018a). As such, ELISA use for individual diagnosis has been discouraged in recent years, though the test still has merit in herd level screening protocols. A recent Australian article has shown that an indirect IgG ELISA test (MilA ELISA) developed by Wawegama at al. (2014) did have better sensitivity when compared to the BIOX K302 test on the same sample set of serum of young calves (Petersen et al, 2018b). Further research is necessary to evaluate the usability of this new test.

4.1.3 ANTIGEN ELISA AND IMMUNOBLOT

Before the rise of ready to use PCR assays, antigen ELISA and immunoblot assays directly detecting *M. bovis* in clinical samples were proposed as a faster alternative compared to culture, reducing the time till diagnosis from a week to days or even only hours (Infante Martinez et al., 1990; Heller et al., 1993, Sachse et al., 1993). Detection limit of the immunoblot when using monoclonal antibodies generally laid around 5×10^3 CFU/mL, and analysis took 2-3 hours to complete (Infante et al., 2002). An immunoblot detecting *M. bovis* in semen was also developed, boasting the same 5×10^3 CFU/mL detection limit, with a sensitivity of 83.3% and a specificity of 100% (Flores-Gutierrez et al., 2004). A combination technique using the antigen capture technique of antigen ELISA to then

perform PCR on these samples, increasing the sensitivity of said PCR to 2-20 CFU/mL has been described as well (Hotzel et al., 1999).

4.1.4 POLYMERASE CHAIN REACTION (PCR)

When PCR was initially developed, it was usually applied for the identification of *Mycoplasma* species out of already cultured samples. Later on, development of (real time) PCR techniques that could be performed on clinical samples allowed to directly detect *M. bovis* DNA, without the need of culturing beforehand (Cremonesi et al., 2007; Parker et al., 2017b). Real time PCR techniques have reduced the time till a diagnosis can be reached, thus leading to a faster response time on affected farms, seemingly without a reduction of the sensitivity. Detection of 10 CFU/ml in milk is possible, although this detection limit depends on the technique and sample used (Cremonesi et al., 2007). A PCR method optimized by Behera et al. (2018), targeting the *uvrC* gene was called 10^3 times more sensitive compared to normal PCR (Rossetti et al., 2010; Behera et al., 2018). However, recent research has also shown that the agreement between *Mycoplasma* culture and multiplex probe PCR identification on semen or swabs can be a lot lower at only 75% agreement (Parker et al., 2017b). One other downside of PCR is that, as with culture, *M. bovis* needs to be present in the sample in order to detect it, and thus intermittent shedders might escape detection. However, in contrast with culture, *M. bovis* does not need to be alive for PCR based detection (Caswell and Archambault, 2008). PCR is a more expensive technique compared to culture, which has resulted in sample pooling in practice (Murai et al., 2014).

4.2. DIAGNOSIS AT HERD LEVEL

Historically mainly culture of animal samples or bulk tank milk was available to assess the prevalence of *M. bovis* in a herd (Sachse et al., 1993). However, as described above, this requires a specialized medium and time. Next to this, the sensitivity of culture on bulk tank milk (BTM) is affected by the intermittent excretion of *M. bovis* in milk, the dilution of the number of bacteria in the whole tank and the fact that the milk of clinical *M. bovis* shedders is usually withheld from the tank (González and Wilson, 2003; Fox et al., 2005). Sensitivity of a single BTM culture for the detection of *M. bovis* infected herds was found to be between 33-59% when at least one cow in the herd was positive for *Mycoplasma*, with the concentration of bacteria not being predictive of the percentage of shedders (González and Wilson, 2002; González and Wilson, 2003). Culturing at least 3 BTM

samples 3-4 days apart will result in a 70% probability of the milked cows being uninfected if all samples test negative (González and Wilson, 2002; González and Wilson, 2003). In herds affected by *M. bovis* pneumonia, 64-90.4% of all nasal swabs tested positive for the bacterium (Soehnlen et al., 2012).

The development of commercial antibody ELISA and PCR tests have facilitated *M. bovis* screening in animals, as they are less time-consuming compared to culture, and both techniques have been validated for use on BTM and composite milk samples as well (Cai et al., 2005; Nielsen et al., 2015; Petersen et al., 2016; Parker et al., 2017a). The sensitivity and specificity of the most commonly used commercially available ELISA on BTM is estimated to be 60.4% (95% CI: 37.5-96.2) and 97.3% (95% CI: 94-99.8) respectively when using the suggested ODC% of 37% (Bio-X BIO K 302, Bio-X Diagnostics, Rochefort, Belgium) (Nielsen et al., 2015). The specificity can be raised (with a decline in sensitivity) by using an ODC% of 50%, as suggested by Nielsen et al. (2015)

However, before the BTM sample will test positive by use of AbELISA, 30% of the lactating animals in a herd need to produce antibodies against *M. bovis* (Petersen et al., 2016). After a clinical mastitis outbreak, antibodies can generally stay detectable in BTM for up to 8 months, but antibody positive young stock does not influence the BTM optical density measurement, making it hard to detect a youngstock outbreak by use of this method (Petersen et al., 2016; Parker et al., 2017a). Because the BTM antibody level fluctuates, it is possible that the antibody levels in BTM drop faster than this as well (Petersen et al., 2016). PCR has a higher sensitivity, but relies on the active excretion of *M. bovis* (Sachse et al., 2010). BTM was the only sample type where PCR could detect more *M. bovis* positive samples compared to culture (Parker et al., 2017b). As *M. bovis* is shed intermittently, and the milk of mastitic cows is supposed to be withheld from the BTM, this could however lead to an underestimation of the *M. bovis* herd prevalence when relying solely on a single PCR analysis (Petersen et al., 2016). Interestingly, PCR and Ab ELISA results showed no agreement, except what could be explained by chance in a study by Parker et al. (2017a). This could mean that using both tests in parallel could raise the sensitivity of correctly identifying positive herds.

5. EPIDEMIOLOGY

Epidemiology studies the distribution and determinants of disease and the application of these factors in the control of said diseases. Especially in the case of *M. bovis*, epidemiological studies are of utmost importance since vaccination is currently unavailable and prognosis is poor. As such, in this section, we delve into the importance of *M. bovis* in Europe, the transmission of *M. bovis* between animals, the currently identified risk factors at herd level and the relevance of molecular epidemiology of *M. bovis* in research and the field.
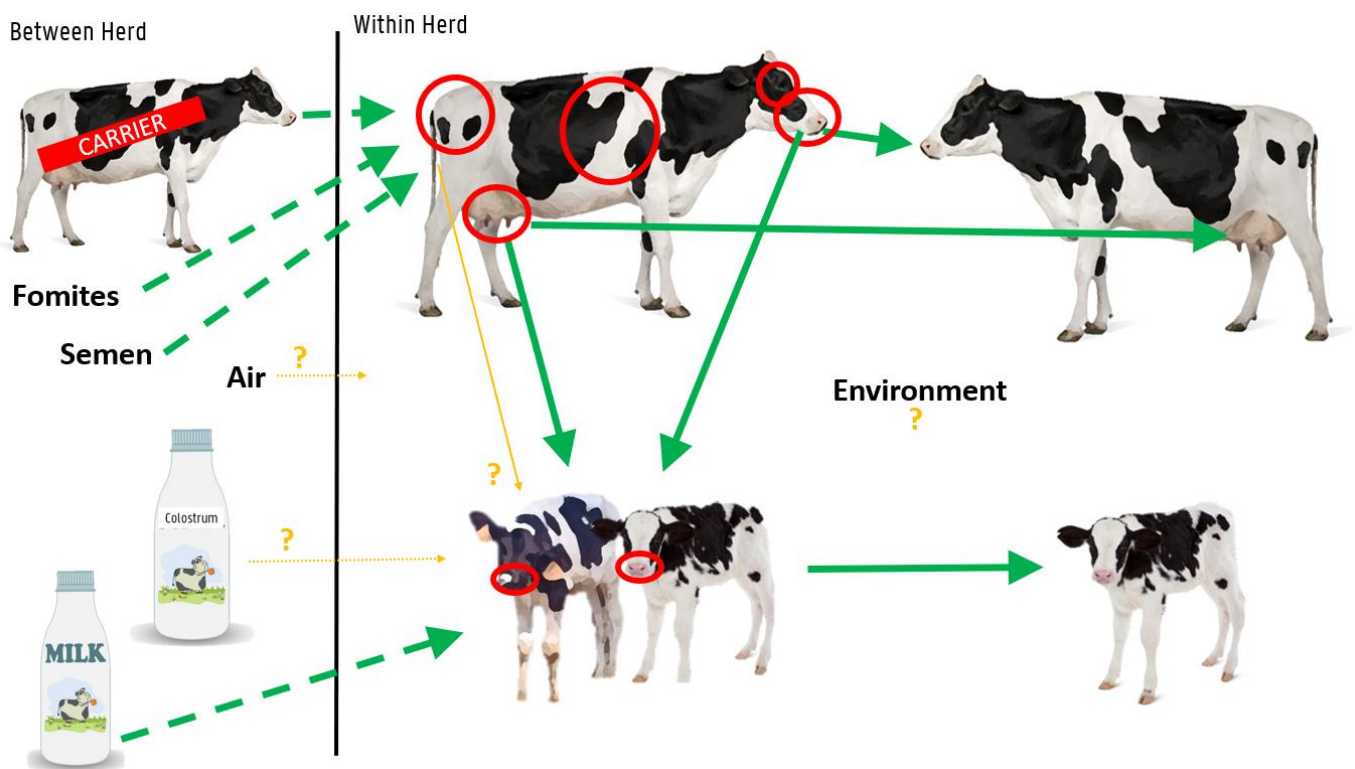
Figure 8: Possible pathways of *M. bovis* transmission. Dashed lines signify between herd transmission, full lines within herd transmission. Orange lines have been suggested but need further research.

5.1. *M. BOVIS* IN EUROPE

After the identification of *M. bovis* in California in 1962, testing of bovine samples gradually started and the geographical spread of *M. bovis* was mapped (Hale et al., 1962; Reeve-Johnson, 1999). *M. bovis* was isolated throughout North America (1962), Israel (1964), Canada (1976), Europe (probably after introduction from America) (1971-1981) and Japan (1977) (ter Laak et al., 1992; Ball, 1999). In early years the prevalence of the pathogen seemed to be quite low and concentrated in veal and beef herds (ter Laak et al., 1992; Reeve-Johnson, 1999). In 1992, the first report was made on the rising prevalence

of *M. bovis* in Europe by ter Laak et al. (1992), who saw an increased incidence of pneumonia, mastitis and arthritis in the Netherlands in the years before this report. In the last decade, *M. bovis* has become a common finding in pneumonia cases in Western Europe. In Italy, 76% of beef cattle and 100% of veal calves presenting with pneumonia at slaughter were found to be carriers of antibodies against *M. bovis* (Radaelli et al., 2008). Similarly, veal operations in France and Belgium also tested around 100% positive for *M. bovis* antibodies (Arcangioli et al., 2008, Pardon et al., 2011).

In Northern Ireland, *M. bovis* got established in 1993, probably imported from mainland Europe after a relaxation of border regulations within the EU (Brice et al., 2000). Between 1993-1998, 15% of all pneumonia cases in Northern Ireland tested positive for *M. bovis*, reflecting the findings in the Republic of Ireland as well, where between 1995-1998, 18% of all pneumonic lungs tested positive (Brice et al., 2000; Byrne et al. 2001). *M. bovis* associated mastitis cases were barely seen during this time. Also in the next follow up period, between 1999-2005, there were hardly any cases of *M. bovis* mastitis found, though the prevalence of *M. bovis* in pneumonia rose to 20% (Blackburn et al., 2007). In Hungary, *M. bovis* was isolated early on, in 1977, and in 2004 11.3% of all animals were seropositive (Fodor et al., 2017). A recent serological study performed in 2017 on 86 herds throughout Hungary unveiled that all Hungarian herds tested had at least 2/10 animals serologically positive, with 88.38% of all herds having more than 50% seropositive animals (Fodor et al., 2017).

Several European countries managed to steer free of the disease until recently (Härtel et al., 2004; Gulliksen et al., 2009; Spergser et al., 2013). Austria had, for example, until 2007 only one confirmed case of *M. bovis* mastitis (Spergser et al., 2013). In 2007, *M. bovis* was found in a large herd, causing devastating disease of cattle and even pigs housed in the same farm (Spergser et al., 2013). The following years the same strain spread to further Alpine areas, causing more animal losses (Spergser et al., 2013). Finland and Sweden stayed free of the disease for a long time as well, but were found to be positive in 2012 and 2011 respectively, albeit at very low prevalence (SVA, 2018; Haapala et al., 2018). A full overview of all known *M. bovis* prevalence levels at herd level in Europe, determined by either ELISA, PCR or culture is depicted in figure 9. In Belgium, two studies determined the herd level prevalence of *M. bovis*, finding 1.5% of all dairy herds positive on culture of BTM in 2009, and 11% of the calves sourced from diverse dairy herds serologically positive (Passchyn et al., 2012; Pardon et al., 2015). Early on, mainly the respiratory

component of *M. bovis* was seen as a problem, especially in veal herds, but more recently the number of *M. bovis* related mastitis cases has risen enormously (Passchyn et al., 2012). PCR analysis on 577 (mainly BAL and DNS) samples collected between 1 December 2016 and 31 October 2018 in Belgian herds suffering from a respiratory disease outbreak resulted in 28.4% of the samples having *M. bovis* DNA presence confirmed (DGZ, 2018).
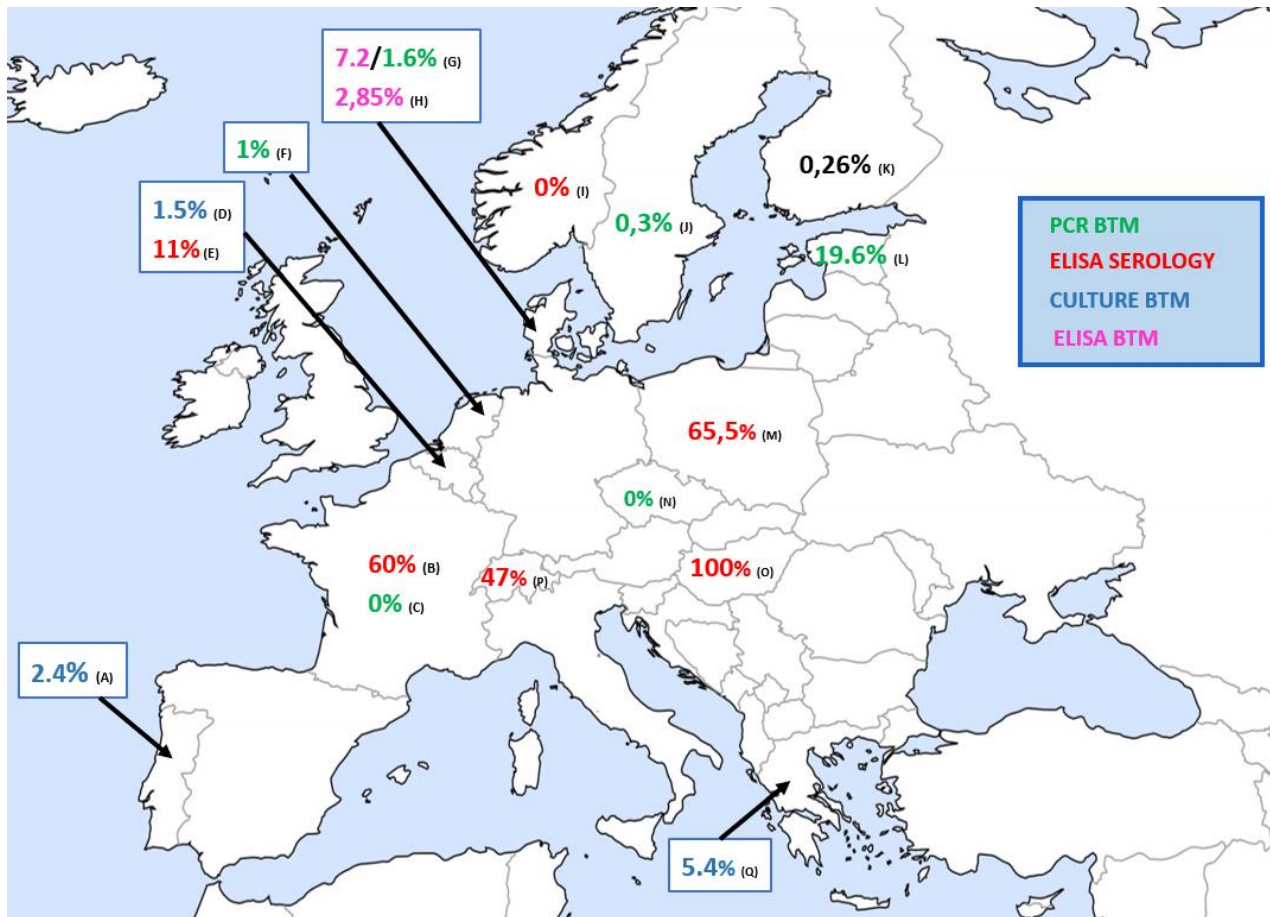
Figure 9: Herd-level *M. bovis* prevalence in Europe determined by use of different techniques

Prevalence data on this map were gathered by a thorough search of various e-libraries, and were published in literature or online between 2002 and 2018. The colors correspond with the type of test used: green stands for PCR on bulk tank milk, red for serological ELISA, blue for culture of bulk tank milk, and pink for ELISA on bulk tank milk. Black stands for a prevalence number mentioned without distinction of the method to obtain said number. **A**: Pinho et al. (2013); **B**: Le Grand et al. (2002); **C**: Arcangioli et al. (2011); **D**: Passchyn et al. (2012); **E**: Pardon et al. (2012); **F**: Hogenkamp (2017); **G**: Nielsen et al. (2015); **H**: Arede et al. (2016); **I**: Gulliksen et al. (2009); **J**: SVA (2018); **K:** Haapala et al. (2015); **L**: Timonen et al. (2017); **M**: Bednarek et al. (2012); **N**: Surýnek et al. (2016); **O**: Fodor et al. (2017); **P**: Burnens et al. (1999) ; **Q**: Filioussis et al. (2007)

5.2. TRANSMISSION

Purchase of a (sub)clinical carrier animal *M. bovis* is generally believed to be the major route of introduction into a herd (Maunsell et al., 2011). Once inside, this carrier will cause infection of other animals, immediately or once shedding is resumed, after which the pathogen will spread through the infected farm (Pfützner and Sachse, 1996). Stress, as induced by transport and handling, can increase shedding in nasal secretions, possibly worsening the initial spread from the introduced carrier animal (Caswell and Archambault, 2008). Stress factors (such as transport, overcrowding, moldy feed and disease) in either adult or calf groups were determined to be a herd-level risk factor for *M. bovis* as well (Aebi et al., 2015). Between adult cattle, the most commonly accepted pathways of *M. bovis* transmission are direct contact, contact with aerosols and transmission during the milking process (Jasper et al., 1974b; González and Wilson, 2003; Maunsell et al., 2011). Calves will usually get infected by consumption of milk contaminated with *M. bovis*, or via direct contact with other carriers (Pfützner and Sachse, 1996; Maunsell and Donovan, 2009; Maunsell et al., 2012).

Several other pathways of transmission have been suggested in the past, such as vertical transmission from the mother to the unborn calf and transmission via colostrum, vaginal secretions, fomites, semen, aerogenous spread and transmission out of the environment. Identification of all possible infection routes and their relative importance is necessary for the design of effective biosecurity protocols and *M. bovis* management directives. In the next paragraphs an overview of current evidence of these suggested routes of transmission is provided.

5.2.1. VERTICAL TRANSMISSION, VAGINAL SECRETIONS, SEMEN AND EMBRYOS

Vertical transmission was reported as a possible route of transmission after isolation of *M. bovis* out of uteri, aborted foeti and neonati (Stalheim and Proctor, 1976; Pfützner and Sachse, 1996). When abortion was induced by inoculation of *M. bovis* into the amniotic fluid, the bacterium could be isolated out of the foetus (Stalheim and Proctor, 1976; Bocklisch et al., 1986). *M. bovis* was shown to be present in the lungs of a preterm calf that died of respiratory problems a few hours after birth as well (Hermeyer et al., 2012). However, the prevalence and importance of vertical transmission remains a question. Since *M. bovis* can be found in vaginal swabs and aborted feti, the assumption can be made that *M. bovis* induced abortions or premature births could be a source of infection for

other cows in the herd. Thus far however, to the author's knowledge, no research has been done on this topic. In the tracing of *M. bovis'* point of entrance in New Zealand, the trade of embryos to be used in embryotransfer was also taken into consideration (Ministry for Primary Industries, 2017). Although *M. bovis* has been isolated from embryos *in vitro* before and commonly used antimicrobials were shown to be ineffective in eliminating the bacterium, the potential of transmission through embryo transplantation remains currently unknown (Bielanski et al., 1989; Bielanski et al., 2000).

Semen can be a carrier of *M. bovis*, and when artificial insemination (AI) semen is stored in liquid nitrogen, *M. bovis* can stay infectious for years (Jasper et al., 1974a, b; Pfützner, 1984). Since the usual combination of antimicrobials (gentamycin, tylosin, lincomycin and spectinomycin) added to AI semen was shown to be ineffective for *M. bovis*, the transmission from this semen to inseminated cattle is in theory possible (Pfützner and Sachse, 1996; Visser et al., 1999), and was shown to be practically possible after outbreaks in closed dairy herds in 2016 in Finland (Haapala et al., 2018).

5.2.2. ENVIRONMENT

M. bovis has the capacity to stay present on a variety of substrates, thanks to biofilm formation (McAuliffe et al., 2006). Among the substrates where *M. bovis* was isolated from, there are a lot of bedding materials, such as straw, manure and recycled bedding sand, which lead to the hypothesis that the environment could be factor in the transmission of *M. bovis* between animals or even production cycles (Pfützner, 1984; Wilson et al., 2011; Piccinini et al., 2015). A complete list of known substrates and their survival time can be found in table 3.

There is however no definite proof that *M. bovis* can transmit to naïve animals from these bedding materials. In fact, Wilson et al. (2011) could prove with 97-99% certainty that recycled sand bedding was not a source of transmission in an experimental setting using a top dressing layer of infected sand in calf pens. Piccinini et al. (2015) have suggested that environment was the source of an infection with *M. bovis* in a veal herd, due to *M. bovis* isolates of calves and environment being "the same molecular (=strain) type". However, this might be a misinterpretation of the data, as *M. bovis* has been shown to spread clonally when introduced into veal herds, with one strain getting the upper hand, which could mean that the environment got infected by the calves, and not vice versa (Soehnlen et al., 2012; Timsit et al., 2012). The survival of *M. bovis* in the environment was

thought to have played a part in the transmission on a farm in cases of mastitis outbreaks described by Bray et al., where cooling ponds (used in summer by milking cows to avoid overheating) were found to be infected with the bacterium and cattle got mastitis after bathing in the infected ponds (Bray et al., 1997; Bray et al., 2001). In any case, the concentration of *M. bovis* found in for example bedding sand (10^3-10^6 CFU/g) is a lot higher than the minimum infective dose necessary to cause mastitis under experimental conditions (as low as 70 CFU/g) (Justice-Allen et al., 2010).

Table 3: Survival of *M. bovis* on various materials present in a barn environment

Tested material	*M. bovis* presence confirmed in barn	Temperature	Survival time at given temperature	Source
Cotton	no	20°C	5 days	Pfützner, 1984
Dirt calving pen	yes	ND	ND	Bray et al., 1997
Manure	yes	23-28°C	236 days in dark / 145 in light	González and Wilson, 2003, Justice-Allen et al., 2010
Metal cages and mangers	yes	ND	2 days	Pfützner,1984, Piccinini et al., 2015
Milk	yes	4°C / 20°C	54 days / 10 days	Pfützner, 1984
Paper	ND	4°C / 30°C / 37°C	126 days / 28 days / 14 days	Nagatomo et al., 2001
Sand	yes	15-20°C	8 months	Justice-Allen et al., 2010
Sponges	ND	20°C	9 days	Pfützner, 1984
Straw	yes	20°C	10 days	Pfützner, 1984, Justice-Allen et al., 2010
Tap water	no	20°C	8 days	Pfützner, 1984
Water of a cooling pond	yes	ND	ND	Bray et al., 1997, Bray et al., 2001
Well water	no	ND	18-20 days	Pfützner, 1984
Wood	no	20°C / 23-28 °C	17 days / <1 day	Pfützner, 1984, González and Wilson, 2003
ND= not determined				

5.2.3. AIRBORNE SPREAD

One study by Jasper et al. (1974a), who laid agars open in a stable and collected them afterwards, has described the isolation of *M. bovis* out of stable air. However, a more specialized study by Soehnlen et al. (2012) using air samplers failed to isolate *M. bovis* out of stable air. Spread in close quarters such as (overstocked) barns could be a risk, especially when housing different ages together, as inhalation of aerosolized *M. bovis* was shown to cause clinical disease (Maunsell et al., 2011; Kanci et al., 2017). Spread by air over longer distances as described for *Mycoplasma hyopneumoniae* in pigs has however probably no or very little importance for *M. bovis* spread (Nicholas et al., 2002; Otake et al., 2010; Kanci et al., 2017).

5.2.4. MILKING EQUIPMENT

Mycoplasma mastitis can easily be transmitted from one udder to the next through contaminated milking equipment, reuse of towels or dirty hands or gloves as well (Jasper et al., 1974b; González and Wilson, 2003). Since only a few *M. bovis* CFU are enough to induce mastitis when introduced via the teat canal, hygiene during milking time and post milking teat asepsis are of utmost importance to prevent the spread of disease (Jasper, 1982; González and Wilson, 2003). Contaminated udder treatments (either reuse of antimicrobial applicators or contamination through improper storage or handling) and unhygienic teat disinfection were shown to be causes of *M. bovis* infection in the past (González et al., 1992; González and Wilson, 2003).

5.2.5. VISITORS AND FOMITES

When the first documented outbreak of *M. bovis* mastitis took place in 1962-1963, *M. bovis* was suspected to have spread between farms due to the visiting veterinarian, as it could be cultured from metal syringes and treatment materials (González et al., 1992).

As *M. bovis* can be present in manure and bedding materials, all herd visitors, especially those visiting multiple farms such as milk truck drivers, merchants and veterinarians and all equipment shared between farms should be seen as an infection risk for the herd (González et al., 1992).

5.2.6. COLOSTRUM

Colostrum was suggested as a source of *M. bovis* infection for calves, but no research was ever published providing definite proof to this claim (Walz et al., 1997; Godden et al., 2006; Foster et al., 2009).

5.2.7. OTHER ANIMAL SPECIES

Since *M. bovis* can, on occasion, infect other animal species and humans it is not unlikely that reinfection to cattle is possible (Madoff et al., 1979; Pfützner and Sachse, 1996; Spergser et al., 2013). However, no definite proof of this pathway is available either.

5.3 HERD LEVEL RISK FACTORS FOR PRESENCE OF *M. BOVIS*

5.3.1 PURCHASE

Purchase is, without a doubt, the main risk factor when looking at *M. bovis* introduction at herd level (Jasper, 1981; González et al., 1992). This is no surprise, since *M. bovis* is often carried by non-clinical shedders, making it difficult to identify on sight (González and Wilson, 2003). Furthermore, any other kind of herd movement where animals of different herds are gathered, such as trade shows and livestock expositions, should be seen as a risk of transmission, and animals should be quarantined after returning to the herd (González and Wilson, 2003; Aebi et al., 2015). Interestingly, Aebi et al. (2015) who found that animal movement (trade and exposition) could raise the odds of having a *M. bovis* detectable presence by 8.3 on univariable analysis, also found purchase of replacements not to be a risk factor for *M. bovis* detection in already exposed herds, except as a source of stress (transport). This could however be a result of the study setup and the small statistical power, combined with the relatively low prevalence of *M. bovis* in the study area (Aebi et al., 2015). In herds already having *M. bovis* present, introduced cattle will also be at risk of acquiring *M. bovis* associated disease (Nicholas et al., 2016).

5.3.2 HERD SIZE

In the years after *M. bovis'* first identification as a cause of mastitis in the USA, herds with a larger number of animals seemed to have a higher incidence of *M. bovis* related disease (Nicholas et al., 2016). In 1981, Thomas et al. found a significant correlation between herd size and culling percentage with *M. bovis* mastitis in Californian herds, but they were unable to make a distinction between cause and effect for culling, whether herds with *M. bovis* had a higher culling rate, or herds with a higher culling rate had more purchase, and thus more risk to having imported *M. bovis* into a herd. A correlation between larger herds and *M. bovis* detection in the herd was also seen by Uhaa et al., (1990), Fox et al. (2003), Pinho et al. (2013) and McCluskey et al. (2003). Herd size was however not observed as a risk factor in a study conducted by González et al. (1992) in the state of New York between 1972 -1990.

5.3.3 SEASON

Seasonal variation in *M. bovis* outbreaks was seen in American herds, where *Mycoplasma* mastitis was more prone to be present from late fall till spring, with a peak around January (González et al., 1992). This seasonality was suggested to be caused by improper ventilation in barns during the winter months (Jasper, 1982). In a large retrospective study, there was a significant seasonal distribution in otitis cases as well, with the highest number of cases being present in spring, and the lowest in summer (Lamm et al., 2004). Interestingly, this seasonal variation was not seen in a large study in Great Britain that analysed a 10 year period of *M. bovis* associated disease (1995-2005) (Nicholas et al., 2008).

5.3.4 WITHIN HERD MANAGEMENT

In a small scale Swiss study, a variety of milking process related risk factors were evaluated as possible risk factors for *M. bovis* positive PCR tests on composite milk samples or nasal swabs (Aebi et al., 2015). In the above study, after univariable analysis, high mean milk production of the herd, forestripping, additional stimulation before milk letdown and a certain milking machine brand were all determined to be potential risk factors for herd-level *M. bovis* presence (Aebi et al., 2015). High mean milk production was suggested to be a source of stress, possibly by causing cattle to be in a negative energy balance, whereas the increased handling of teats before milking could have been a source of pathogen transfer between animals (Aebi et al., 2015).

The use of a sick pen to isolate animals out of the main herd instead of immediately culling *M. bovis* mastitis affected animals was shown to raise the odds of other cows in the pen getting infected, but the complete lack of a sick pen has also been identified as a risk factor (Punyapornwithaya et al., 2011; Fox, 2012). Cows returning from the sick pen to the main herd should be followed closely for signs of mastitis or mycoplasmosis, to avoid having too many shedders in the main herd (Nicholas et al., 2016). Herds with methods in place to identify problem cows (color markers, leg tags) were shown to have less risk of having detectable *M. bovis* in the BTM (Pinho et al., 2013). In veal herds, the mixing of age groups was shown to be a significant risk factor for seroconversion to *M. bovis*, with animals showing severely reduced weight gain during seroconversion and a severe increase in antimicrobial use (Tschopp et al., 2001).

5.4 WITHIN HERD CIRCULATION

Once *M. bovis* has entered a herd, transmission will happen between animals. However, the degree of transmission will vary in time. After the initial mastitis outbreak caused by a single strain in a previously non infected herd, Punyapornwithaya et al. (2010) found 33.5% of all (mostly nonclinical) animals positive on one or more body sites with culture in the first three months after the outbreak. This decreased to 1.4-5.6% of the animals testing positive in the next year of sampling without any more incidences of *M. bovis* mastitis (Punyapornwithaya et al.,2010). In an Estonian cross-sectional study, 17.2% of all animals of a single herd (n=522) were found to be positive on PCR for *M. bovis* DNA on individual composite milk samples at a single time point (Timonen et al., 2017). In veal calf rearing facilities, nasal colonization by *M. bovis* was shown to happen quickly after the introduction of the calves into the herd, with 90% of all animals having had at least one nasal swab positive at 100-120d of age (Soehnlen et al., 2012).

5.5 MOLECULAR EPIDEMIOLOGY

Molecular epidemiology is becoming an invaluable tool to determine the origin of *M. bovis* strains and the route or source by which they entered a specific farm or region (Nicholas et al., 2016). By strain typing, one can also determine whether a strain is herd specific, whether different strains cause different types of disease, and even (through DNA sequencing) whether specific loci are predisposing for antimicrobial resistance or strain dependent disease (Nicholas et al., 2016). Multiple methods have been developed, but interpretation and comparison between studies and laboratories is difficult (McAuliffe et al., 2004; Pinho et al., 2012; Nicholas et al., 2016).

Older techniques are still in use, and are especially useful as methods to compare novel techniques with previous studies (Pinho et al., 2012). Examples of such techniques are amplified fragment length polymorphism (AFLP) analysis, a DNA fingerprinting technique based on PCR that generates band profiles via selective amplification of restriction fragments of the whole genomic DNA, random amplified polymorphic DNA (RAPD) analysis, which uses short primers that by use of PCR amplify different fragments of the genome which are then separated and visualized by gel electrophoresis, and pulsed field gel electrophoresis (PFGE), which uses a restriction enzyme to digest the DNA, followed by a separation of the fragments using two alternating electrical fields. AFLP, RAPD and PFGE were compared in a large scale study on strains from the UK, with AFLP and RAPD showing the best congruence (McAuliffe et al., 2004). PFGE has been used often

in past studies, but is being replaced by other methods due to its time-intensive, specialized and costly methodology and low level of discrimination between strains (Pinho et al., 2012). Insertion sequence typing (IS typing) is another, more discriminating, typing technique using mobile genetic elements (insertion sequences) present in *M. bovis'* DNA (Miles et al., 2005; Aebi et al., 2012). In general, all of the above techniques are very hard to reproduce and lab-specific, making comparison difficult (Pinho et al., 2012).

After the full genome of *M. bovis* was sequenced, multiple new methods were developed based on this information (Pinho et al., 2012). Multilocus Sequence Typing (MLST) is one of those methods, detecting single point mutations in housekeeping genes to compare and determine the relationship between *M. bovis* strains (Register et al., 2015; Rosales et al., 2015). This technique is repeatable and protocol is the same between laboratories, making it a possible candidate for worldwide application. Two different protocols have been proposed using different housekeeping genes (Register et al., 2015; Rosales et al., 2015), with the one by Register et al. (2015) being used as the reference protocol for the PubMLST database. However, this protocol was recently shown to be of limited use, as one of the targeted housekeeping genes was shown to be absent in an important part of the *M. bovis* population, making differentiation between strains very difficult (Communication at the ruminant meeting of the International Organization for Mycoplasmology, Portsmouth, 2018). Multiple-locus variable-number tandem-repeat analysis (MLVA) is another technique, based on the full genome of *M. bovis* PG45, using 9 different tandem-repeat sequences to compare genetic microvariations, with results comparable to RAPD and PFGE and generally more discriminating then MLST (Pinho et al., 2012; Sulyok et al., 2014; Becker et al., 2015). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) is an upcoming technique, capable of rapidly identifying bacteria after isolation (Pereyre et al., 2013). Whereas this technique is mainly used for species identification, prospective studies have shown that strain typing to subspecies level might be possible, but further research is needed on this topic (Pereyre et al., 2013; Becker et al., 2015).

Studies have shown that *M. bovis* strains derive from multiple genetic clusters. In a study on 54 strains collected from respiratory disease in Great Britain over 6 years, McAuliffe et al. (2004) saw two distinct genetic clusters using RAPD and AFLP, and a lot of variability in between strains. These findings were confirmed more globally with IS-typing and MLST

by others (Miles et al., 2005; Rosales et al., 2015). Within a country, clusters may exist as well, and in countries with low *M. bovis* circulation, a single strain might be the cause of multiple outbreaks, such as in the Austrian Alps *M. bovis* outbreak from 2009-2011, where 96.8% of the isolated strains had the same profile using both RAPD and MLVA (Spergser et al., 2013). Using MLST, Lysnyansky et al. (2016) found that 60% of all mastitis outbreaks in Israel between 2004-2014 were caused by a single strain, with a rise in genetic variability in recent years, possibly related to import. The same difference in genetic composition between strains was seen by Amram et al. (2013) when comparing strains from imported calves suffering from pneumonia and mastitic cows. A study by Becker et al. (2015) comparing *M. bovis* isolates obtained during a 35 year period by use of MLST, MLVA and MALDI-TOF MS found with all three techniques two clusters as well, separated in time. More recent strains (isolated after 2000) had a reduction in the diversity of the isolates, indicative of a single clone spreading throughout the country, possibly related to acquired antimicrobial resistance that emerged in the same timespan (Gautier-Bouchardon et al., 2014; Becker et al., 2015). Within a farm, there is a tendency for only one genetic profile to be present (McAuliffe et al., 2004; Aebi et al., 2012; Sulyok et al., 2014). However, this might be influenced by the degree of herd movements: whether cattle is purchased, traded or temporarily removed from the farm (eg. trade shows, heifer rearing) might influence the number of strains present at any given time (McAuliffe et al., 2004). Sulyok et al. (2014) also reported finding only one *M. bovis* strain in farms when comparing them with MLST. However, when using a more discriminating method (MLVA), within-farm strain differences were seen, indicating the possibility of closely related strains circulating within a farm.

Strain typing by use of whole genome sequencing such as suggested for other pathogens might prove possible in the future. This would be a more discriminating method compared to for example PFGE in *M. bovis* strain typing as well. (Salipante et al., 2015; Wise et al., 2001).

6. TREATMENT AND THERAPY FAILURE

6.1. ANTIMICROBIAL TREATMENT

In the past, it has been stated that mycoplasmosis is resistant to treatment with any chemotherapeutical agent (Pfützner and Sachse, 1996). Especially *Mycoplasma* mastitis is seen as a cause for culling and treatment is strongly discouraged (Pfützner and Sachse, 1996; Nicholas et al., 2016). Some classes of antimicrobials (mainly macrolide and phenicol derivates) were however shown to have significant effect on the reduction of *M. bovis* induced respiratory disease and weight loss when used in experimental settings or field experiments (Godinho et al., 2005; Catry et al., 2008; Bartram et al., 2016; Lysnyansky and Ayling, 2016). Antimicrobial treatment of otitis media has even shown to result in a clinical recovery rate of 75% (Gosselin et al., 2012). Until vaccines are available, the use of antimicrobials is the only possible intervention after introduction of *M. bovis* into the herd.

Only a few classes of antimicrobials have a known bactericidal effect on *M. bovis*, namely the (fluoro)quinolones and (at high doses) the aminoglycosides such as neomycin and gentamicin (Lysnyansky and Ayling, 2016). All other antimicrobial classes are bacteriostatic, slowing growth to allow the body to kill the bacteria (Lysnyansky and Ayling, 2016). Following recent guidelines concerning the overuse of antimicrobials in veterinary medicine, fluoroquinolones should be used sparingly and only when the bacteria are resistant to other antimicrobials given their critically important status in human medicine (WHO, 2017; AMCRA, 2018). Spectinomycin, florfenicol and tulathromycin were shown to have an effect on *M. bovis* induced pneumonia in experimental studies or field trials (Poumarat et al., 2001; Godinho et al., 2005; Catry et al., 2008). Of these, florfenicol is seen as the first treatment choice in Belgian national guidelines (AMCRA, 2018). As such, this agent should be used first, with as a second choice chlortetracycline, doxycycline, oxytetracycline, tilmicosin, tulathromycin or tylosin (AMCRA, 2018).

6.2. ANTIMICROBIAL RESISTANCE

Due to the absence of a cell wall, *M. bovis* is naturally resistant to all β-lactam antimicrobials (Chernova et al., 2016). Next to this, *M. bovis* does not synthetize folic acid, which means antimicrobial products of the sulfonamide class will not be effective (Maunsell et al, 2011). Further innate resistance for polymyxins, trimethoprim, nalidixic

acid and rifampicin has been described for other mycoplasmas (Lysnyansky and Ayling, 2016). Even when using a supposedly effective antimicrobial, therapy failure happens often, due to multiple reasons (Bushnell, 1984). As mentioned above, *M. bovis* has the capacity to "hide" intracellularly, effectively evading antimicrobials (Burki et al., 2015). Due to the chronic nature of the lesions induced by *M. bovis*, the bacteria are able to "hide" in the necrotic lesions as well, making it hard for the administered antimicrobials to reach the site of infection (Kleinschmidt et al., 2013). Furthermore, in recent years, more and more loss of antimicrobial susceptibility has been reported (Ayling et al., 2014; Gautier-Bouchardon et al., 2014).

The rise of (multi)resistant bacteria is a major concern worldwide (WHO, 2017). The development of antimicrobial resistance in *M. bovis* seems to be mainly related to mutations in chromosomal genes (Lysnyansky and Ayling, 2016). Different techniques have been described for antimicrobial susceptibility testing such as microbroth dilution, agar dilution and diffusion, flow cytometry and E-tests. In recent years DNA testing has become available, checking the presence of different loci predisposing for antimicrobial resistance (Gautier-Bouchardon, 2018; Sulyok et al., 2018). Currently, microbroth dilution methods are still used as the main method of susceptibility testing of *Mycoplasma spp.*, usually based on guidelines set out by Hannan (2000). The determination of MIC values through microbroth dilution methods is time consuming and specialized, making it hard to perform in a routine fashion, and different laboratories often use different media or color indicators making comparison between studies difficult (Sulyok et al., 2018). A novel real-time PCR molecular assay developed by Sulyok et al. (2018) seems promising, detecting mutations on genome level responsible for elevated MICs of fluoroquinolones, tetracyclines, aminocyclitols, macrolides, lincosamides, phenicols and pleuromutilins in *M. bovis*. This technique could reduce the time to produce a susceptibility profile from weeks to 3-4 days after initial isolation of the bacteria. However, having genetical markers for resistance does not always effect in a phenotypical resistance, and this technique will only work for these markers already identified.

Antimicrobial resistance of a certain bacterium is often hard to define. Two main criteria are used to distinguish between susceptible and resistant bacteria: the use of clinical break points, and the use of epidemiological cutoff values (Dung et al, 2008; Schwarz et al., 2010). Clinical breakpoints, as set by the Clinical and Laboratory Standards Institute

(CLSI) or the Veterinary Subcommittee on Antimicrobial Susceptibility Testing (VetCAST) are therapeutic breakpoints, based on *in vitro* tests, pharmacokinetic data, *in vitro* resistance markers, outcomes of clinical studies and other results (Dung et al., 2008; Maunsell et al., 2011; Toutain et al., 2017). They are used to predict the therapeutic success of a certain antimicrobial to a certain bacterium, dividing the tested strains into sensitive, intermediate and resistant categories. Sadly, no CLSI or VetCAST clinical breakpoints are available for veterinary *Mycoplasmataceae* to this date (Rosenbusch et al., 2005; Maunsell et al., 2011; Toutain et al., 2017). The microbiological or epidemiological cutoff is another criterion, distinguishing between strains which are part of the "wild type" population, and those which have acquired resistance to the antimicrobial tested by evaluating the distribution of the determined susceptibility levels graphically (Dung et al., 2008; Schwarz et al, 2010).

The determination of minimum inhibitory concentrations (MIC) will give an indication of the *in vitro* susceptibility of the tested strain, but this might not be directly related to the *in vivo* susceptibility and the subsequent possibility of therapeutic success (Nicholas and Ayling, 2003; Schwarz et al, 2010). Even though there are no clinical breakpoints available for *M. bovis*, several studies have aimed to determine the susceptibility of *M. bovis* strains to various antimicrobials, and have used various methods to attempt to interpret which drugs strains were *in vivo* resistant to, and which drugs were still recommended for treatment (Ayling et al., 2014; Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016).

In Europe, even before 2000, tetracycline and macrolide resistance was already reported (Gautier-Bouchardon, 2018). The overall antimicrobial susceptibility of *M. bovis* to commonly used veterinary antimicrobials was found to be diminishing when compared to older strains as well, both in France, Great Britain and the Netherlands (Ayling et al., 2014; Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016). Interestingly, the antimicrobial susceptibility of *M. bovis* seems to vary between countries (Klein et al., 2017). In the Netherlands, fluoroquinolones, tulathromycin and oxytetracycline were suggested to still be the most efficacious (Heuvelink et al., 2016). In France however, tulathromycin and oxytetracycline were among the antimicrobials with the highest increase in MIC50 and were even interpreted as being completely impotent against all strains tested (Gautier-Bouchardon et al., 2014). Strain sensitivity in Israel was different between native and imported *M. bovis* strains in a study by Gerchman et al. (2009),

indicating country specific selection pressure as well. Due to the absence of clinical breakpoints, it is possible that strains interpreted as being resistant will still have a clinical effect: for example, when tulathromycin was used to treat clinical disease caused by a strain (*M. bovis* isolate 956, originally isolated from a BRD case in Italy) that tested apparently resistant *in vitro*, a detectable impact could still be seen in some cases in a study conducted by a pharmaceutical company (Bartram et al., 2016).

M. bovis seems to adapt quickly to antimicrobial selection pressure, as illustrated by its resistance to the first choice antimicrobials in multiple countries (Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016). Furthermore, resistance was shown to be predilection site dependent. For example, pneumonia strains showed a higher MIC to tulathromycin, an antimicrobial only indicated to treat respiratory disease, compared to strains isolated from mastitis lesions in one study (Heuvelink et al., 2016; Gautier-Bouchardon, 2018). However, in a small British study, this was the opposite, finding mastitis strains to be more resistant compared to respiratory strains (Ayling et al., 2014).

Generally, given the chronicity of *M. bovis* induced lesions, the intrinsic resistance against several antimicrobials and the rise in MIC values of other antimicrobials, prevention of *M. bovis* induced disease would be much more effective than antimicrobial use. However, on an industrial scale, the rapid use of antimicrobials to rein in a pneumonia outbreak might be necessary to reduce losses, with the understanding that it will likely not result in a 100% cure rate (Nicholas and Ayling, 2003).

6.3. INTERACTION OF *M. BOVIS* WITH OTHER THERAPEUTIC AGENTS

Dexamethasone was shown to have a positive effect on edema reduction and reinitiating milk production when used in cases of mastitis. However, the effect proved only temporary, and a more severe remission was seen after halting treatment (Bushnell, 1984). Even more, dexamethasone administration was shown to increase shedding of *M. bovis* in calves and to have a synergistic immunosuppressive action combined with *M. bovis* (Alabdullah et al., 2015; Alabdullah et al., 2018; Calcutt et al., 2018).

7. PREVENTION AND CONTROL

As illustrated in the previous chapter, therapy of *M. bovis* induced disease is difficult and often fails. Prevention of the disease and control of spread in affected farms should be the primary focus.

7.1 VACCINATION

Multiple research groups have tried, with little avail, to create an effective vaccine to protect herds against *M. bovis* or to reduce the damage of *M. bovis* induced disease (Pfützner and Sachse, 1996; Nicholas et al., 2002; Maunsell et al., 2009; Mulongo et al., 2013; Dudek et al., 2016). Even though small scale experiments often seem to have protective effect reducing gross pathological lesions and increasing *M. bovis* specific antibody titers, large scale studies of commercially available vaccines have shown no- or little protection or even an increase of lesions caused by *M. bovis* in vaccinated animals (Nicholas et al., 2002; Maunsell et al., 2009; Soehnlen et al., 2011; Mulongo et al., 2013). Strain differences and the variable expression of Vsps could explain this limited efficacy (Dudek et al., 2016). The variability in the expression of the Vsps poses a big difficulty, as they are on the one hand very immunogenic, making them an ideal candidate for use in a vaccine, but on the other hand, because of their variable expression, Vsp based vaccines might not stay effective for long (Lysnyansky et al., 1999; Perez-Casal et al., 2017). Research focused on the use of conserved recombinant proteins instead of bacterin vaccines is now developing, in the hope of providing better results in large-scale trials (Perez-Casal et al., 2017). Autogenous vaccination, where a herd-specific strain is inactivated and used in the same herd, might be a small scale solution in closed herds, but will have limited success in herds with frequent intermingling such as feedlots and efficacy might be short lived, due to the frequent changes in the Vsps (Perez-Casal et al., 2017). Live-attenuated vaccines have shown promise as well, but will need further in-depth research before they can be implemented on large scale (Zhang et al., 2014).

As long as vaccine-based protection is unavailable, current *M. bovis* management programs should be based on the control of present infections and the prevention of *M. bovis* spread in and between herds.

7.2 PREVENTION OF INTRODUCTION

Especially *M. bovis* free herds should try to abstain from purchasing cattle as this is the largest risk factor for introduction of the bacteria (Maunsell et al., 2011). If cattle are

purchased, a quarantine period should be respected, during which testing of the purchased animals is advised through serology, nasal swabs or milk analysis (González and Wilson, 2002; González and Wilson, 2003). In the case of dairy herds, analysis of multiple BTM samples of a prospective herd before purchase might be prudent as well (Maunsell et al., 2011). When a previously uninfected herd is found to be positive, stamping out policies have been tried before (Pfützner and Sachse, 1996). However, the success of these hinges on how early *M. bovis* was detected.

Next to purchase, the removal and subsequent reintroduction of animals into herds such as in the case of trade shows, off site rearing, summer grazing etc. should be seen as a risk. Purchase or introduction of any biological materials which could be a carrier of *M. bovis* (eg. milk, colostrum, faeces, AI semen, embryos) into negative herds should be avoided as well, due to its inherent infective potential. Furthermore, due to the persistence of the bacteria in the environment, all farming equipment and transportation devices used on multiple farms need to be disinfected thoroughly before introduction. Lastly, herd visitors in contact with multiple farms or cattle derived from multiple farms such as veterinarians, milk truck drivers, animal merchants etc. and all fomites in contact with animals (medication, clothing, sampling devices, …) should be seen as a hygienic risk.

7.3 CONTROL MEASURES ON AFFECTED FARMS

On dairy farms, due to its chronicity, treatment failure and the possible presence of shedders, many authors have advised to cull all animals that test positive for *M. bovis* on culture or PCR (González and Wilson, 2003; Nicholas et al., 2016). Bulk tank milk testing can be used as a screening method. When the tank tests positive, identification of the shedders should be attempted (González and Wilson, 2003). Since subclinically affected cattle often have a high SCC and a decreased milk production, these cows need to be looked at closely, combined with recently calved cows and clinical mastitis cases (Maunsell et al., 2011; Al-Farha et al., 2017; Timonen et al., 2017). If culling is not an option, affected animals need to be separated from the normal milking herd and milked last, while respecting rigorous hygienic measures (Punyapornwithaya et al., 2012). However, in the hospital pen, the introduction of *M. bovis* positive cows can lead to an increase in transmission of the disease, possibly leading to even more animals getting infected and becoming carriers (Punyapornwithaya et al., 2011). Milking hygiene and especially individual udder preparation is of utmost importance to prevent the spread

during the milking process. Gloves should be worn while milking, disinfecting them between animals (Jasper, 1982; Bushnell, 1984). Teats should be dipped with 1% iodine or commercial teat dip after cleaning (to remove biologic and organic materials), and milking equipment should be disinfected after each turn by backflushing or disinfection of the teat cups with spray washing or rinsing with sanitizer (González and Wilson, 2003). Housing in poorly ventilated barns, and having calves and adult cattle housed together, seems to predispose for more mastitis cases, especially when at least a group of animals is suffering from respiratory disease as well (González et al., 1992; González et al., 1993).

To prevent the spread of *M. bovis* to young calves, several preventive measures can be taken. Since milk was shown to be a prime carrier of *M. bovis*, calves should preferably be fed with milk replacer or pasteurized milk in herds suffering from *M. bovis* disease (Bennett and Jasper, 1978; Walz et al., 1997; Maunsell et al., 2012). In any instance, waste milk or milk from mastitic cows should not be fed to calves (González and Wilson, 2003). Tank milk can be pasteurized at 65°C for 1 hour to effectively eliminate the threat of transmission (Butler et al., 2000). Acidification of milk to a pH of 4 or lower for at least 1 hour also effectively inhibited *M. bovis* growth, but this treatment might cause other practical problems such as reduced palatability, incubation time and separation of the milk in fractions (Parker et al., 2016).

Colostrum has been called a *M. bovis* infection source as well, but there has been no definite proof of this (Walz et al., 1997; Godden et al., 2006). Pasteurization can decontaminate colostrum, and commercial gamma irradiated colostrum is available as well (Godden et al., 2006). Care should be taken to not overheat the colostrum, as the antibodies present within are heat sensitive (McMartin et al., 2006). Pasteurization of 30-60 minutes at 60°C is effective to kill all *M. bovis* present, without a discernable effect on the immunoglobulin G (IgG) content, but the consistency will be altered (Godden et al., 2006; McMartin et al., 2006). Lyophilized colostrum can be used as an alternative, if a high enough total amount of immunoglobulins is provided (Klobasa et al., 1998).

Individual housing is another very effective measure to avoid rapid spread of *M. bovis* between calves through nose-nose contact (Caswell et al., 2010; Maunsell et al., 2011). For individual housing, it is imperative to prevent contact between calves (eg. by housing them in calf igloos) and to not swap feed or milk buckets between animals. Since aerosols, lack of ventilation and overcrowding of stables were also shown to be possible infection

sources or predisposing factors, housing outside might be preferable (Maunsell et al., 2011). In group housing, an all-in all-out approach is preferred, since this will prevent contact between the younger, more sensitive calves and the older calves (Nicholas and Ayling, 2003). Chronically infected calves, growth stunted calves and especially clinically ill animals (eg. with a head tilt, fever, runny nose or arthritis) should not be housed together with healthy animals to prevent the spread of *M. bovis* in the herd (Maunsell et al., 2011). Chronically ill cattle should be separated and closely followed, if they do not put on weight, euthanasia should be considered as an option to safeguard animal welfare and reduce infection pressure (Caswell et al., 2010). The prognosis for chronically infected calves is poor: Caswell et al. (2010) noted a positive predictive value of 50-75% for euthanasia at the time of entry into the chronically infected pen.

As *M. bovis* can stay present in the environment for days to months, disinfection of the environment should be considered when dealing with an outbreak (Pfützner, 1984; Justice-Allen et al., 2010). In the case of bedding sand, use of 0.5% sodium hypochlorite or 2% chlorhexidine was sufficient to completely kill all *M. bovis* present (Justice-Allen et al., 2010). Also heat and other commonly used disinfectants such as chlorine-, acid- or iodine based disinfectants are effective against *M. bovis* (Maunsell et al., 2011). *M. bovis* is sensitive to desiccation, even though biofilm formation might be protective (at least in part), so leaving the disinfected pens to dry out might further reduce environmental *M. bovis* contamination (Justice-Allen et al., 2010; Bürki et al., 2015).

8. RESEARCH GAPS

Currently, the absence of an effective vaccine is seen as one of the biggest problems in *M. bovis* prevention. However, due to the chronic nature of the disease and the presence of shedders, this is not the only problem we are faced with. Some of *M. bovis'* disease presentations and subsequent possible shedding sites might have been neglected in the past, which is especially important when looking at closed herds or areas with no to little *M. bovis* circulation. Due to the increase in global trade and the amount of bovine related products traded between farms (eg. AI semen, embryos, second hand machinery), the infectious potential of these products needs to be evaluated. Furthermore, the uncertainty concerning a possible aerogenous spread, environmental spread or spread through water should be elucidated as well. In general, focus should lay on the prevention of disease, as such, the identification of more risk factors predisposing for disease or spread of *M. bovis* are sorely needed. This also encompasses the determination of infectious doses for various infection routes.

Updated, generalized European prevalence data should be determined for *M. bovis* pneumonia and mastitis alike, to be able to monitor the risk of importing cattle from a specific country. To reduce and manage the disease and its impact on farm economics and cattle welfare in the future, we urgently need to extend our knowledge on its epidemiology.

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CHAPTER 2

SCIENTIFIC AIMS

In the last decades, the apparent increased incidence and spread of *Mycoplasma bovis* into previously naive countries has worried the scientific community. More recently, the enormous economic burden, large impact on antimicrobial use and reduced animal welfare have alarmed farming communities, veterinarians and governments as well. Mycoplasmosis has been extensively described, but a lot of research gaps in *M. bovis'* epidemiology and predilection sites still remain. Information on national prevalence data is lacking as well. Given the absence of an effective treatment or vaccine, prevention of further spread of the disease is a key priority. Therefore, the overall objective of this doctoral thesis was to fill in gaps in the current knowledge of *M. bovis'* epidemiology, its predilection sites and possible routes of transmission.

The specific objectives of the present thesis were:

(1) To determine the prevalence of *M. bovis* in Belgian dairy farms, and to identify herd level-risk factors associated with a positive bulk tank milk sample (Chapter 3)

(2) To gain insight on the link between *M. bovis* and colostrum (Chapter 4), by assessing the survival of *M. bovis* in colostrum through freezing (Chapter 4.1) and by determining the prevalence of *M. bovis* DNA in colostrum samples (Chapter 4.2).

(3) To describe a new predilection site, seromas, and to use molecular typing techniques to assess within animal and between herd spread of the causal strain (Chapter 5)

CHAPTER 3

PREVALENCE AND RISK FACTORS OF

MYCOPLASMA BOVIS IN DAIRY HERDS

USE OF A BREEDING BULL AND ABSENCE OF A CALVING PEN AS RISK FACTORS FOR THE PRESENCE OF *MYCOPLASMA BOVIS* IN DAIRY HERDS

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ABSTRACT

Mycoplasma bovis is an important cause of pneumonia and mastitis in cattle throughout the world, often reported as emerging. In absence of an effective vaccine for *M. bovis*, current prevention and control strategies rely on the identification of risk factors for within and between herd spread. The objective of this study was to determine the prevalence of *M. bovis* in Belgian dairy herds and to identify risk factors associated with a positive PCR and/or Ab ELISA bulk tank milk (BTM) test.

A cross-sectional study was performed in 2016 on 100 dairy farms, analyzing the BTM using PCR and antibody ELISA. Information on herd level risk factors focusing on biosecurity and management were collected through a questionnaire and sourced from the national herd identification system (SANITRACE). Multivariable logistic regression was used to identify herd-level risk factors for the presence of *M. bovis* DNA and antibodies in the BTM. The apparent prevalence on BTM was 7% and 17% for PCR and antibody ELISA, respectively. The true prevalence was 7.1% (95% Confidence interval (CI): 2.1-11.5%) and 24.8% (95% CI: 16.4-33.2%). There was no overlap between ELISA and PCR positive farms, resulting in a combined true prevalence of 31.8% of the Belgian farms being in recent contact with *M. bovis*.

Risk factor analysis showed that herds with a breeding bull (*M. bovis* positive results for 45.5 % and 13.6% of herds with and without a bull respectively, odds ratio: 4.7 (95% CI: 1.1-19.8)) and without a calving pen (*M. bovis* positive result in 52.4% and 20.6% of the herds without and with a calving pen, respectively, odds ratio: 3.7 (95% CI:1.06-12.5)) had higher odds to harbor *M. bovis* antigen or antibodies in the BTM. In conclusion, the present study points to a several fold increase in the prevalence of *M. bovis* in Belgian dairy herds. The importance of the breeding bull and calving pen in the between- and within-herd spread of *M. bovis* might have been underestimated in the past. Focusing on these factors might contribute to more effective control programs in the future.

INTRODUCTION

Mycoplasma bovis is the *Mycoplasma* species with the highest economic impact in cattle (Nicholas and Ayling, 2003). Highly contagious, it can cause a variety of clinical presentations of which (chronic) pneumonia, arthritis and otitis in calves and mastitis and pneumonia in adult cattle are the most common (Maunsell et al., 2011).

Historically only culture has been available to assess the prevalence of *M. bovis* (Sachse et al., 1993). The development of commercial antibody (Ab) ELISA and PCR tests has facilitated further *M. bovis* screening in animals. Ab ELISA and PCR have been validated for use on bulk tank milk (BTM) and composite milk samples, serving as an easy to use indicator for the presence of *M. bovis* in a herd. (Cai et al., 2005; Nielsen et al., 2015; Parker et al., 2017). The sensitivity and specificity of a commercially available ELISA (Bio-X BIO K 302, Bio-X Diagnostics, Rochefort, Belgium) on BTM is estimated to be 60.4% (95% CI: 37.5-96.2) and 97.3% (95% CI: 94-99.8) respectively (Nielsen et al., 2015). Approximately 30% of the lactating animals in a herd need to produce antibodies against *M. bovis* before the BTM sample will test positive on ELISA (Petersen et al., 2016). After a clinical outbreak the antibodies stay present in BTM for about 8 months (Parker et al., 2017). PCR has a higher sensitivity, but relies on the active excretion of *M. bovis* (Sachse et al., 2010). As *M. bovis* may be shed intermittently, and the milk of mastitic cows is supposed to be withheld from the BTM, this could lead to an underestimation of the *M. bovis* herd prevalence when relying solely on a single PCR analysis (Petersen et al., 2016).

Current approaches for *M. bovis* control are highly variable and based on the available knowledge of *M. bovis* spread (Maunsell et al., 2011). Between herds, the biggest *M. bovis* introduction risk is attributed to the purchase of carrier animals. Between adult animals, the milking process and direct contact are seen as the main causes of transmission (Maunsell et al., 2011). In calves, feeding of milk from infected cows (such as feeding waste milk) is seen as an important cause (Maunsell and Donovan, 2009). Other causes of transmission such as via fomites, airborne or aerosol spreading and colostrum have been suggested (Godden et al., 2006; Gille et al., 2016; Calcutt et al., 2018). Recently, infected semen was linked to the introduction of *M. bovis* into two closed herds (Haapala et al., 2018). Elimination of *M. bovis* is very difficult to impossible. Especially in the case of *M. bovis* mastitis, experts urge to separate and cull affected cattle instead of trying to treat (González and Wilson, 2003; Fox et al., 2005; Nicholas et al., 2016). Since treatment

options are limited, prevention on herd and cattle level alike is key. Given the current lack of a useable vaccine (Perez-Casal et al., 2017), prevention hinges on the identification and elimination of epidemiological risk factors for *M. bovis* infection. Unfortunately, only a limited number of risk factors have been identified to this date. Having a larger herd size showed a strong association (15 times higher odds) with having mycoplasma in the BTM (Thomas et al., 1981). In the same study, a small association was also observed between a high culling rate and a positive BTM was seen. González et al. (1992) however, could not find an association between herd size and mycoplasmal mastitis. A study by Burnens et al. (1999) found only the purchase of animals was significantly linked to the serological *M. bovis* status of a herd (OR: 10.8). Aebi et al. (2015) found that farms applying forestripping, having a high average milk production or a lot of herd movements had higher odds of having *M. bovis* present. The objectives of this study were to investigate the prevalence of *M. bovis* in dairy herds in Belgium by determining the presence of *M. bovis* DNA and antibodies in BTM samples and to identify risk factors for a positive BTM sample.

MATERIALS AND METHODS

A cross-sectional study was conducted on 100 dairy herds in Flanders (Belgium) in January 2016. The study population was randomly selected (aselect procedure in Microsoft Excel) from the national cattle identification database (SANITRACE, animal health service Flanders). The herd selection was stratified on province according to cattle density. Sample size calculation was done through WIN EPISCOPE (Win Episcope 2.0, Zaragoza, Spain). A sample size of 97 herds was calculated from the pool of approximately 6600 Flemish dairy herds (Belgium, 2016), using a worst case *M. bovis* prevalence of 50%, with 80% power and 95% confidence. BTM samples were collected during routine milk sampling by the Flemish milk control center (MCC-Vlaanderen) over the course of January 2016. All farms currently not enrolled in the milk quality control program of MCC were automatically excluded from this study. In 2016, 4628 dairy herds were enrolled in the program, where 6597 herds in total produced milk in Flanders (Belgium, 2016, MCC, 2016). Samples were cooled (4-8°C) and immediately transported to the laboratory for analysis.

Table 1: Questionnaire provided to the farmers to gauge the internal and external biosecurity and general herd management concerning *M. bovis*: Overview on the data collected by questionnaire.

All questions were yes vs. no questions unless otherwise specified between brackets.

Subject	Description
M. bovis exposure	Previous positive tests on the farm; Known colleagues with problems
Herd movements/ Exposure to other herds	Trade show visits; Calf rearing expedited; Neighboring pastures with cows; Use of farming machines from other herds; Use of farming equipment from other herds; Trading of bulls between farms; Distance to nearest neighbor herd (in m); Months of pasture access for young stock, heifers, cows; Use of a purchase protocol (blood testing); Use of a quarantine period (Yes, >2 weeks/ Yes, maximum one week/ No)
Environmental factors	Drink water type (rainwater/city water/well water)
Hygienic measures	Herd visitors (farm specific clothing/ farm specific boots/ boot disinfectant/ disinfection of car/ none); Bird control measures; Rodent control measures; Cleaning of calf pens (removal of straw and feces/ high pressure cleaning/ disinfection); Separate calving pen (Yes, one cow at a time/ Yes, multiple animals at a time/ No); Use of calving pen by sick animals
Calf rearing and internal biosecurity	Use of milk of another herd for calf rearing; Use of colostrum of another herd; Use of lyophilized colostrum; Use of gamma-irradiated colostrum; Calf disease representation in the last year (Cough/ head tilt/ arthritis/ umbilical infections/ diarrhea/ pneumonia/ none of the above); Colostrum providing (Bottle/ tube/ suckling); Individual housing (Igloo inside/ igloo outside/ individual box in a separate young stock stable/ individual box in adult stable); Milk (cow's milk/ powder milk/ suckling calves); Age of grouping (in weeks); Group housing (outside/ box in young stock stable/ box in stable of adult cows); Use of an automatic milk feeder; Calf contact when individually housed; Individual drinking buckets ; Contact of calf caretaker with adult cows
Herd visitors	Amount of visiting veterinarians; Frequency of livestock-dealer visits (in weeks); Visits by people also visiting veal farms
Reproduction	Use of a breeding bull ; Use of a teaser bull

Commercially available real-time PCR (PathoProof Mastitis Complete 16 PCR assay, Thermo Fisher Scientific, Finland) and *M. bovis* antibody ELISA (BIO K 302, Bio-X Diagnostics S.A., Belgium) were performed on the samples according to the manufacturer's instructions. Specifically for the ELISA, the manufacturers' recommended cutoff value of 37% OD was used to guarantee the best combination of sensitivity and specificity. After determining the apparent prevalence, true prevalence was calculated using Epi Tools (Ausvet, 2018).

To collect information on potential risk factors, a questionnaire containing 44 questions related to internal and external biosecurity and general herd management was distributed to all farms by email (Table 1). The questionnaire was made available online through Google Forms. Non-responders were interviewed by telephone. All interviews were done by the same researcher. Interviews were conducted in the second part of 2016. Further herd information (mean herd size, amount of purchase, mortality) was gathered from the national registry of cattle movements (SANITRACE). All questionnaire data were put into a spreadsheet (Microsoft Excel) and transferred to SAS 9.4. (SAS Institute Inc., Cary, Inc.) for statistical analysis.

To identify risk factors associated with the detection of *M. bovis*-specific DNA or antibodies in BTM, a multivariable logistic regression model was built (PROC LOGISTIC). The outcome variable was a PCR and/or antibody ELISA positive result. The elementary unit was the farm. In a first step, potential risk factors were tested univariably for their association with the outcome variable. After univariable analysis, variables with a P value of 0.10 or less were withheld for multivariable analysis. Predictors were grouped and recoded if an insufficiently low number of cases was present in a given category. Several factors could not be included in the univariable analysis due to a too low number of observations. Correlation between different predictors was tested with Pearsons and Spearman rho correlation. If the correlation was above 0.6, only the most significant variable was withheld for further analysis. Multivariable analysis was performed stepwise backwards, gradually excluding non-significant variables (P<0.05). Biologically relevant interactions between significant main factors were tested. Model fit was checked by the Hosmer-Lemeshow goodness of fit test. Significance was set at P≤ 0.05 and P≤ 0.10 was considered a trend.

Table 2: Results of univariable analysis of factors associated with an *M. bovis* positive test result on BTM: All variables with *P* ≤ 0.10.

Variable	Category	*n*	% Neg	% Pos	*P*-value
Separate calving pen	No	21	47.6	52.4	0.02
	Yes	34	79.4	20.6	
Individually housed calves	No	2	0	100	0.04
	Yes	53	69.8	30.2	
Purchase protocol used when purchasing cattle	No	19	84.2	15.8	0.05
	Yes	36	58.3	41.7	
Use of a breeding bull	No	22	86.4	13.6	0.01
	Yes	33	54.5	45.5	
High pressure cleaning of calf pens	No	25	56.0	44.0	0.07
	Yes	29	79.3	20.7	
Colostrum feeding by tube	No	50	72.0	28.0	0.02
	Yes	5	20.0	80.0	
Otitis (head tilt observed by farmer)	No	50	64.0	36.0	0.10
	Yes	5	100	0	
Purchase of cattle	No	22	86.4	13.6	0.02
	Yes	33	54.5	45.5	
Neg: negative BTM sample, Pos: PCR or Ab ELISA positive result, BTM: Bulk Tank Milk					

RESULTS

Prevalence and herd characteristics

Overall, 7% (95% CI: 2.06 – 11.49%) of the farms tested positive by PCR on BTM, with a true prevalence of 7.1% (95% CI: 2.06-11.49%). Seventeen percent of all farms tested Ab ELISA positive on BTM (95% CI: 9.72 – 24.28%). The true prevalence was 24.8% (95% CI: 16.42-33.15%) for the ELISA results. None of the farms that tested positive in PCR had detectable antibodies present in the bulk tank milk and none of the farms that were Ab ELISA BTM positive were PCR positive.

The response rate of the questionnaire was 55% (55/100). Of these, 5 farms (9%) were PCR positive and 13 farms (23,6%) ELISA positive. The mean herd size of the farms that filled in the questionnaire was 157 (range: 62 - 460 animals), which was not significantly different from the target population (mean: 134) but did trend to be among the larger herds (G=1.93; df =53; p=0,06).

No milk from other herds was purchased and no bulls were shared with other herds by any of the 55 herds. Only one herd used a teaser bull for heat detection. None of the herds used automated milk feeders. All calves were housed inside after weaning. All of these factors were excluded from further analysis given the low number of observations in one or more categories.

Concerning biosecurity, only one of the herds applied hygienic measures to clean off premise vehicles. One fourth of all interviewed farmers indicated that they did not enforce any hygienic measures for visitors at all. When farmers used a bull for some animals, but not the whole herd (eg. to breed rebreeders), they were grouped in the "uses a bull for insemination" category. Sixty percent of the farms still used a bull on some or all cows.

Risk factor analysis

Due to the relatively small number of returned questionnaires, the outcome variable was adapted to "having a *M. bovis*-specific DNA or antibody positive BTM sample" instead of analyzing data from ELISA positive herds separately from PCR positive herds. After univariable analysis eight variables with a *P* value of 0.10 or less were withheld for multivariable analysis: the use of a separate calving pen, individual housing of calves, the use of a cattle purchase protocol (serological testing of a variety of diseases at purchase), the use of a breeding bull, high pressure cleaning, colostrum feeding by tube, presence of

otitis media and purchase in the year before testing (Table 2). Purchase of cattle in the year before testing was significantly correlated to the presence of a bull, but the bull was more significantly linked to a positive sample. The final model consisted of two significant risk factors for a positive BTM result after multivariable analysis (Table 3). Farms which used a breeding bull had 4.7 higher odds to test positive. The use of a separate calving pen was a protective factor (OR= 0.27).

Table 3: Final multivariable model for *M. bovis* PCR or antibody ELISA positive bulk tank milk samples

Variable	Category	Herds (n)	% Positive	Odds Ratio	95% Confidence interval		*P*-value
Use of a breeding bull	No	22	13.6	Ref.			
	Yes	33	45.5	4.7	1.1 - 19.8		0.04
Separate calving pen	No	21	52.4	Ref.			
	Yes	34	20.6	0.27	0.08 - 0.94		0.04

DISCUSSION

This study aimed to determine the prevalence of *M. bovis* in Belgium, and to identify new risk factors. One hundred dairy farms were randomly selected, stratified on province.

Selection bias cannot be ruled out, since herds needed to participate in the milk control program in order to be eligible for this study. It is possible that these herds are, in general, larger and possibly more modern. In the present study a relatively high response rate of the questionnaire of 55% was achieved. However, this still left the study with relatively low power due to the initial sample size, which could mean certain risk factors remain unidentified. Since the questionnaire was made available online, and non-responders were contacted by phone, a certain measure of observer bias was possible. However, to mitigate this, only one researcher conducted the interviews by telephone, following the same questionnaire as what was made available online. Due to time constraints interviews were performed more than 6 months after the initial sampling. This might have been a cause of recall bias in the interviewees, although none indicated to have done a big shift in farming in the time passed. Only one sample per herd was taken, which could have impacted the sensitivity of this study (Biddle et al., 2003).

In Belgium in 2009, 1.5% of all BTM samples from three consecutive samplings in 200 herds tested positive using bacterial culture (Passchyn et al., 2012). In this study, done on the same sample pool in 2016, 7% of the tested herds had active circulation of *M. bovis*, detected through a PCR positive BTM sample. This seems to be a notable increase especially since only one sample per herd was taken. However, comparing the two studies is difficult due to the difference in technique. When comparing to recent studies determining between-herd prevalence using PCR on BTM from the Netherlands (approx. 1% in 2017) (Hogenkamp, 2017), Denmark (1.6 % in 2015) (Nielsen et al., 2015) and the south-east of France (0% in 2011) (Arcangioli et al., 2011), our findings seem to indicate a higher active infection rate in Belgian farms compared to other closeby countries. One possible explanation for this higher prevalence could be the intensive contact structure (40% of all cattle born between 2005-2009 moved herds at least once (Ensoy et al., 2014)) and high geographical density of Belgian herds. In contrast to PCR, Ab ELISA does not detect active circulation, but can detect recent contact of a herd with *M. bovis*. The true prevalence using Ab ELISA on BTM in this study was 24.8%. Since this BTM ELISA technique is relatively new, only data of one other country, Denmark, is available to this

date. Nielsen et al. (2015) found a prevalence of 7.1% in BTM of Danish herds when using the same test with the recommended cutoff of ODC% 37%. Comparing BTM ELISA results with previous serological studies on *M. bovis* prevalence is difficult: Parker et al. (2017) found a relatively low correspondence between serological results and BTM ODC%, suggesting a lower usability of the BTM test as a between-herd screening tool when trying to predict within-herd seroprevalence. Antibodies were detectable in the BTM for up to 12 months after the initial outbreak. After this period, despite having further positive *M. bovis* culture or PCR results, the BTM ODC% dropped under the detection limit. However, notwithstanding these limitations, in our opinion BTM analysis can still be a valid between-herd screening tool in the field, for example in a purchase protocol, especially when combined with PCR analysis as it is practical and low cost. Previous research found virtually no overlap between PCR or ELISA positive BTM samples, except what could be explained by chance (Parker et al., 2017). This reflects our findings in the current study, where no overlap was seen between PCR and ELISA samples. A possible hypothesis of this lack of overlap is that, in newly infected herds, PCR positive samples will appear at least 1-2 weeks before seroconversion, by which time it can be expected that actively infected animals have shown signs of mastitis and are separated from the BTM (Nicholas et al., 2002). Next to this, it is possible that *M. bovis* antibodies are developed in response to other *M. bovis* associated diseases such as pneumonia or arthritis, without subsequent shedding in milk. Regardless of the test, the results in this study seem to indicate a higher *M. bovis* prevalence level compared to neighboring countries.

One of the most interesting observations of this study was the association between the presence of a breeding bull and a *M. bovis* positive BTM sample (DNA or Ab). Moreover, where previous purchase was identified as a risk factor for the presence of *M. bovis* in a herd (Burnens et al., 1999), this study showed that, although purchase was part of the univariable risk factors, it was strongly associated with the presence of a breeding bull, and the breeding bull was the more significant factor of the two. Bulls are still often used in Belgian herds to breed with rebreeders and older cows (as a means to produce more valuable crossbreed calves). Next to the purchase of the bull, Belgian dairy herds are often closed, with year-round calving patterns. Herd size was not significantly linked to *M. bovis* presence on the farm in this study, in contrast with the findings of Thomas et al. (1981) and Fox et al. (2003) but consistent with the findings of González et al. (1992). Since the biggest herd included in this study only had around 460 animals, it is possible that the

Belgian mean herd size is not big enough to be able to distinguish significant differences between smaller and larger herds. Another possibility is that purchase and herd size are linked as well, where larger herds might buy in (or have bought) cattle more often to sustain their growth.

In bulls, *M. bovis* colonizes the prepuce and the distal urethra (Fish et al., 1985), decreasing the fertility (Bielanski et al., 2000). Seminal vesiculitis, epididymitis and persistent infection along the seminal tract with concurrent shedding have been described (Kirkbride, 1987). *M. bovis* has been found in vaginal swabs of apparently unaffected cattle and cattle suffering from clinical mastitis (Punyapornwithaya et al., 2010; Hazelton et al., 2018). It was isolated from uterine samples, out of aborted foeti and in postsurgical seromas after caesarean section (Stalheim and Proctor, 1976; Pfützner and Sachse, 1996; Gille et al., 2016). When AI was performed with *M. bovis* infected semen persistent infection of the genital tract was seen, with shedding for up to 8 months post AI (Hirth et al., 1966). The findings of the present study, suggesting the importance of the bull, combine well with a recent report of *M. bovis* introduction into 2 naïve herds in Finland by use of AI (Haapala et al., 2018). Also in the recent introduction of *M. bovis* in New Zealand, semen was a suspect of being the source of introduction (Ministry for Primary Industries, 2017). *M. bovis* in semen can survive cryopreservation for up to 18 months (Hirth et al., 1966). The commonly used antimicrobial cocktail to decontaminate AI semen could be insufficient (Visser et al., 1999). Given recent observations and our current findings, it seems possible that the role of semen in the *M. bovis* epidemiology has been underestimated in the past.

A second remarkable observation was the protective nature of a separate calving pen for the *M. bovis* status of the herd. There seem to be two possible explanations to this protective effect. On one hand, it is possible that the periparturient immunity depression could induce shedding of higher numbers of *M. bovis* at calving. However, even though *M. bovis* has been isolated out of placenta and fetal fluids, no research has been done on the infectious capacity of *M. bovis* excreted during parturition (Stalheim and Proctor, 1976). On the other hand, the use of a calving pen might protect the immunosuppressed periparturient animals from infection by limiting contact with carriers present in the herd. Limiting contact between susceptible animals and shedders will in any case limit the exposure, with concurrent less shedding in the BTM. An interesting parallel can be drawn between *M. bovis* and *Chlamydia sp.,* as both the breeding bull and calving away

from the herd were shown to be risk factors for the latter as well (Kemmerling et al., 2009).

CONCLUSIONS

A considerable proportion of the Flemish dairy herds had recently been in contact with *M. bovis* at the time of this study. Based on PCR results, the active prevalence of *M. bovis* seems to be higher compared to studies in neighboring countries. This study identified having a breeding bull and the absence of the calving pen as risk factors for having an ELISA or PCR positive BTM sample. Further attention should be given to the role of the breeding bull and calving pen in the spread of *M. bovis* in a herd, and their potential role in the development of effective control and preventive measures for *M. bovis*.

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CHAPTER 4

MYCOPLASMA BOVIS IN COLOSTRUM

EFFECT OF FREEZER STORAGE TIME AND THAWING METHOD ON THE RECOVERY OF *MYCOPLASMA BOVIS* FROM BOVINE COLOSTRUM

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ABSTRACT

Mycoplasma bovis is an important cause of mastitis in dairy cattle, and pneumonia, arthritis, and otitis in calves. Milk and colostrum are considered important sources of infection for calves. Knowledge on the effect of on-farm freezing ($-18°C$) and thawing methods on the recovery of *M. bovis* from colostrum samples is missing. In this study, 2 separate experiments were performed. The first experiment consisted of a longitudinal study examining the survival [as measured by log(10) reduction] of 2 *M. bovis* strains in frozen colostrum over 14 wk. The second experiment examined the effect of different thawing temperatures (45 and 20°C), thawing frequencies (once or twice), and initial colostrum titer (10^4 or 10^6 cfu/mL) on *M. bovis* survival. A single freeze-thaw cycle led to an approximate 1 log reduction of *M. bovis* titer, independent of the thawing temperature. Freezing for 14 wk did not significantly further reduce the titer of bacteria compared with freezing for 2 wk. A second freeze-thaw cycle further reduced the *M. bovis* count by approximately 0.5 log compared with a single freeze-thaw cycle. Thawing temperature and initial bacterial concentration did not significantly affect *M. bovis* reduction. In conclusion, storage of colostrum samples in the freezer at $-18°C$ during epidemiological studies, herd monitoring, or test and cull programs will probably have little influence on qualitative bacteriological test results for *M. bovis*. The epidemiological or clinical relevance of an approximate 1 log reduction of *M. bovis* in colostrum is currently unclear.

Mycoplasma bovis (*M. bovis*) has been recognized as an important cause of untreatable mastitis in adult cattle and chronic, unresponsive pneumonia in calves, frequently complicated by arthritis and otitis (Maunsell and Donovan, 2009; Maunsell et al., 2011). Annual losses due to *M. bovis* have been estimated to be above 140 million dollars in the US (Rosengarten and Citti, 1999). It is generally accepted that the most important way of *M. bovis* introduction into a farm consists of purchase of a carrier (Fox, 2012). Sperm can be infectious (Pfützner and Sachse, 1996), and recent work suggests the possibility of between herd spread through fomites or persons (Gille et al., 2016).

Known routes of transmission at individual animal level are the milking process, direct nasal contact, semen and the consumption of infected milk (Pfützner and Sachse, 1996; Fox et al., 2005). For calves in particular, consumption of infected milk is believed to be the primary route of infection (Walz et al., 1997; Butler et al., 2000; Maunsell and Donovan, 2009; Maunsell et al., 2012). Relatively few studies have documented the within herd prevalence of *M. bovis* in milk samples. In a recent Swiss study the within herd prevalence of *M. bovis* shedding cows was 2.4% (95% confidence interval (CI)= 1.5-3.8) on milk by PCR assay (Aebi et al., 2015). In other studies a within herd prevalence between 2.6 and 43.0% has been reported (Fox, 2012).

Colostrum has been mentioned as a possible source of infection as well, but the prevalence of *M. bovis* in colostrum samples is currently unknown (Godden et al., 2006). Preliminary PCR testing of colostrum samples at the Belgian center for milk quality control (MCC-Vlaanderen) did identify some positive samples (personal communication, K. Supré). The presence of *M. bovis* in colostrum is currently unaccounted for in *M. bovis* preventive protocols. Indeed, the commonly recommended individual housing for 8 weeks without nose-nose contact, together with replacing cow's milk by milk replacer, might have limited efficacy to reduce mycoplasma infection if *M. bovis* positive colostrum has been given previously (Maunsell and Donovan, 2009). Colostrum samples are often stored frozen prior to analysis (Godden, 2008). However, it is unknown how freeze-thaw cycles affect recovery of *M. bovis* from these colostrum samples. Freezing of milk samples has been shown to reduce the recovery of *M. bovis* by culture (Boonyayatra et al., 2010). Considering the different composition of colostrum compared to milk (Foley and Otterby, 1978), simple extrapolation of the survival data of *M. bovis* in milk to colostrum may be incorrect (Boonyayatra et al., 2010).

The objectives of the present study were to determine the effect of the duration of the freezing period, the number of freeze-thaw cycles and the thawing temperature on the survival of *M. bovis* in colostrum samples inoculated with two concentrations of *M. bovis*. Two separate experiments were conducted to achieve these objectives.

The colostrum used in experiments one and two was purchased gamma irradiated frozen bovine colostrum (ECI, Marloie, Belgium), guaranteeing immunoglobulin levels over 70 g/L and a sterile product. Sterility was verified by plating the colostrum on standard blood culture, incubated at 37°C, 5% CO_2 for 24h and on Pleuropneumonia Like Organism (PPLO) agar (DIFCOTM, BD©), incubated for one week, to check for mycoplasmal growth specifically. Colostrum was thawed at room temperature before inoculation with *M. bovis*.

In experiment one, the effect of the duration of the freezing period on survival of *M. bovis* in colostrum was evaluated. Two strains of *M. bovis* were inoculated in colostrum. At inoculation (T0) and after 1 (T1), 4 (T2), 10 (T3) and 14 weeks (T4) of storage at -18°C, three colostrum samples for each strain were thawed at 20 °C (room temperature) and serial culture was performed to determine the *M. bovis* titer.

In experiment one, two strains of *M. bovis* (LG1 and LG2) were used. LG1 originated from a bronchoalveolar lavage sample from a calf with pneumonia, LG2 originated from a milk sample from a cow with mastitis, arthritis and an infected seroma (Gille et al., 2016). *M. bovis* species identification was confirmed by use of real-time PCR targeting the uvrC gene of filter-cloned isolates (Rossetti et al., 2010). In preparation for this study, two hundred microliters of a *M. bovis* strain suspended in a storage medium consisting of 75 ml horse serum (Thermo Fisher Scientific, Carlsbad CA, USA) and 25 ml Brain Heart Infusion (BHI) broth (Bio-Rad, Hercules CA, USA) supplemented with 10% (w/v) glucose (Merck, Darmstadt, Germany) was inoculated into 10 mL of modified PPLO broth (DIFCO™). After inoculation, the broth was incubated for five days at 35°C and 5% CO_2, after which the PPLO broth was centrifuged at 4500 x *g* for 30 minutes to sediment the bacteria. The supernatant was discarded and the pellet was resuspended in sterile phosphate buffered saline (PBS) (M: 0.1; Ph 7.4). Dilution of the bacterial suspension to approximately 10^{10} CFU/mL was done by use of an optical density meter (Ultrospec III, Pharmacia Biotech, UK). The sample was diluted until an optical density of 0.2 absorbance units at 540 nm was achieved, based on specifications by Boonyayatra et al. (2010). Further dilution was done to achieve starting titers of 10^6 *M. bovis* CFU / mL colostrum. The inoculated

colostrum samples were stored in sterile 15 mL Falcon® centrifugation tubes (Fisher Scientific™, MA, US) and frozen at -18°C. *M. bovis* titer was determined immediately after complete thawing (no ice visible). 100 µL colostrum was plated on PPLO agar using serial dilutions. Samples were incubated for one week at 35°C and 5% CO_2, and colonies (with the typical fried-egg appearance) were counted with help of a microscope (10x magnification). Each sample was only plated once, but for each timepoint multiple samples were thawed.

To determine the effect of freezing over a 14 week period and the effect of *M. bovis* strain on the survival of *M. bovis* in colostrum samples a linear mixed model with repeated measures was used (PROC MIXED). Five time points (at inoculation (T0), after 1 week (T1), after 4 weeks (T2), after 10 weeks (T3) and after 14 weeks (T4)) were included as the within-subjects factor, strain type (LG1 vs. LG2) was added as a between-subject factor. The sample size (3 observations per group per time point) was based on the detection of a 1 log difference between both strains, with a standard deviation of 0.4 log, 80% power and 95% certainty. Mauchy's test of sphericity was used to determine equality of variances. Model validity was checked through inspection of the residuals. Bonferroni corrections were used for pairwise comparisons between the different time points. A compound symmetry repeated variance structure was used. In all models significance was set at $P<0.05$, and $0.05<P<0.10$ was considered a trend. All analyses were performed in SAS version 9.4 (USA).

In the second experiment, the effect of repeated thawing (once or twice) and temperature of the first thawing process (either 20°C (room temperature) or 45°C) on *M. bovis* recovery was determined using two inoculum titers (10^4 and 10^6 *M. bovis* CFU/mL). The sample size required to detect a 1 log reduction in *M. bovis* count between two storage/thawing methods, with 95% certainty and 80% power was 18 observations per group (Win episcope 2.0, Spain).

A full factorial design was used with four test groups: (1) ST20 (single thawing 20°C) = freezing for two weeks, single thawing at 20°C; (2) ST45 (single thawing 45°C)= freezing for two weeks, single thawing at 45°C. (3) RT45 (repeated thawing 45°C): freezing for 1 week, thawing at 45°C, refrozen for 1 week, thawing at 20°C; (4) RT20 (Repeated thawing 20°C)= freezing for 1 week, thawing at 20°C, refrozen for 1 week, thawing at 20°C.

Sample preparation was identical to experiment one, except that only the LG1 strain was used, in titers of 10^4 and 10^6 *M. bovis* CFU/mL colostrum. The inoculated colostrum samples were split in 16 portions of 10 mL colostrum, which were distributed over the four treatment groups. The experiment was repeated three times. One week after inoculation, groups RT20 and RT45 were thawed at 20°C and 45°C, respectively, and refrozen at -18°C. All groups were thawed at their respective temperatures 2 weeks after inoculation. *M. bovis* titer was determined using the same method as in experiment 1.
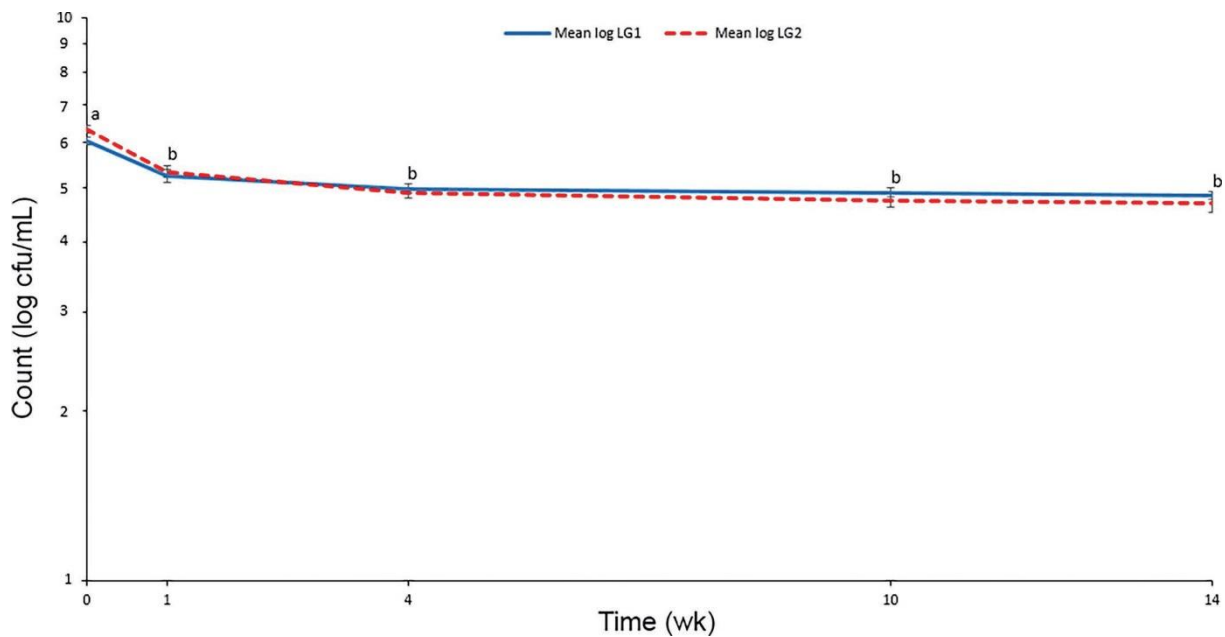
Figure 1. Mean concentration of colony-forming units per milliliter of colostrum after freezing and thawing a single time over a set time period. Time = 0 = inoculation; time = weeks of freezing. Values with different letters (a,b) are significantly different (P < 0.05) within subjects (time effect). Error bars represent SD*.*

A linear mixed-model (PROC MIXED) was used to determine the effects of the initial *M. bovis* concentration in colostrum (10^4 vs. 10^6), number of freeze-thaw cycles (1 vs. 2) and thawing temperature (20°C vs 45°C). The test run was added as a random effect to account for clustering of measurements within a run. A maximum likelihood estimation with Satterthwaite approximation was used. All factors were forced into the model and interactions between significant main effects were tested. Post-hoc comparisons were made using Bonferroni corrections. Model validity checking and significance definitions were as described for experiment one.

In experiment one, the effect of freezing duration on the survival of *M. bovis* in colostrum was determined. Freezing significantly reduced *M. bovis* concentration by a mean of 0.81-

1.02 (SD: 0.13-0.15) log for LG1 and LG2, respectively between inoculation and 1 week later (Figure 1). Longer freezing times did not result in further decrease in the number of *M. bovis* recovered from colostrum. No significant effect of strain was observed (Figure 1). Values with a different superscript were significantly different from each other (P<0.05) within subjects (time effect).

Table 1. Effect of different thawing temperatures and repeated thawing on recovery of *Mycoplasma bovis* from bovine colostrum samples, with a titer of 10^4 or 10^6 CFU/mL *M. bovis.*

Thawing Temp	Low starting titer (10^4 CFU/mL)				High starting titer (10^6 CFU/mL)				*P*-value			
	Single Thaw		Double Thaw		Single Thaw		Double Thaw					
	20°C	45°C	20°C	45°C	20°C	45°C	20°C	45°C	Freq.	Temp.	Conc.	Freq. x Conc.
CFU/mL *M. bovis**	3.55± 0.33	3.73± 0.28	3.18± 0.35	3.14± 0.36	5.80± 0.23	5.65± 0.40	5.18± 0.35	5.0± 0.68	<0.001	0.43	<0.001	0.17

Results presented as means of 3 independently repeated experiments ± standard deviation

*log transformed concentration

Freq.: Thawing frequency, Temp.: Temperature, Conc.: Inoculation Concentration

The effects of repeated thawing and thawing temperature tested in experiment two are shown in Table 1. Independent of the starting titer (10^4 or 10^6 CFU/mL), freezing and thawing of *M. bovis* infected colostrum reduced the *M. bovis* concentration by approximately 1 log compared to the initial concentration. Thawing temperature (20°C versus 45°C) did not have a significant effect on the survival of *M. bovis* in colostrum (P= 0.43). Repeated thawing of colostrum further decreased the number of *M. bovis* by approximately 0.5 log compared to single thawing, regardless of the starting concentration of *M. bovis* (P<0.05).

As expected, freezing and subsequent thawing of colostrum did not result in a complete elimination of *M. bovis*, similar to previous observations in milk samples (Boonyayatra et al., 2010). After a single freeze-thaw cycle, the *M. bovis* concentration was reduced by approx. 1 log, independent of the starting titer, which is comparable to the findings reported for milk samples (Boonyayatra et al., 2010). Unlike the aforementioned study, longer freezing intervals were tested in this study as well. This did not result in a significant further decline of *M. bovis* CFU. Mycoplasma concentration in milk ranges between 10^2 and 10^8 CFU/mL, with the vast majority above 10^6 CFU/mL (Biddle et al.,

2003; Cai et al., 2005). If the *M. bovis* concentrations in colostrum and naturally infected milk are similar, an approximate 1 log reduction will result in *M. bovis* concentrations ranging between 10 and 10^7 CFU/mL colostrum. Such *M. bovis* concentrations can be detected with most of the commonly used screening methods such as PCR, ELISA and culture (detection limit: 10 CFU/mL (Biddle et al., 2003)). Intermittent shedding has been described for several Mycoplasma species, so negative results should be interpreted carefully (Biddle et al., 2003).

Extension of storage time at -18°C for up to 14 weeks did not further decrease the survival rate of *M. bovis* in colostrum, as compared to storage for one week. These results further illustrate that long-term freezing of colostrum is not a valid control strategy to prevent *M. bovis* infection of neonatal calves. Double freeze-thaw cycles resulted in a significantly larger reduction of *M. bovis* in colostrum. Multiple freeze-thaw cycles can cause an undesirable decline in maternal antibody levels (Argüello et al., 2003) and are thus not recommended.

In conclusion, single and double freeze-thaw cycles reduce *M. bovis* concentration in colostrum by 1 to 1.5 log respectively. Thawing temperature and initial bacterial concentration did not significantly affect the survival of *M. bovis* in colostrum. The storage of colostrum samples in the freezer at -18°C during epidemiological studies, herd monitoring or test- and cull programs likely has little influence on qualitative bacteriological test results for *M. bovis*. As there are currently no indications on the minimal infective dose of *M. bovis* in colostrum, the epidemiological or clinical relevance of an approximate 1 log reduction of *M. bovis* in colostrum is currently unclear and deserves further attention in future research. In case a lower *M. bovis* titer reduction is desired for epidemiological studies, addition of glycerol might be a valid aid, as described for milk samples by Boonyayatra et al. (2010).

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PRESENCE OF *MYCOPLASMA BOVIS* IN COLOSTRUM

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ABSTRACT

In herds with an active *Mycoplasma bovis* circulation, colostrum is often considered a source of infection for neonatal calves. Control measures to prevent infection include the removal or treatment of said colostrum, causing economical and practical issues, possibly resulting in a suboptimal start of the calf's life. However, to date no studies on the presence of *M. bovis* in colostrum are available. Therefore, this study aimed to determine the prevalence of *M. bovis* DNA in colostrum samples of herds with a recently confirmed *M. bovis* infection in Belgium. In total, 368 colostrum samples were collected in 2016 and 2017 from 17 farms. Only 1.9% (7/368) of the samples tested PCR positive for *M. bovis*, with 13 out of 17 sampled herds having no detectable *M. bovis* DNA in colostrum. The within herd prevalence averaged 3.2% (standard deviation= 4.9%; Range: 0-30.0%). Only three samples had a Ct-value below or at 37, four others had higher Ct-value (<40), indicating a low concentration of bacterial DNA. In conclusion, with the PCR assays used in the present study, *M. bovis* DNA was occasionally detected in colostrum samples in herds with *M. bovis* circulation. The epidemiological relevance of this observation is currently unknown. The present results may indicate that in infected herds, the within herd transmission due to colostrum is low compared to the other ways of transmission. In *M. bovis* free herds, however, the purchase of untreated colostrum is discouraged to avoid introduction.

Keywords: prevention, biosecurity, calf, epidemiology

SHORT COMMUNICATION

Mycoplasma bovis strongly contributes to economically important diseases like mastitis and pneumonia and heavily affects animal welfare and antimicrobial use in modern dairy farming (Calcutt et al., 2018). Prevalence seems to be rising and increasing antimicrobial resistance has been reported as well (Gautier-Bouchardon et al., 2014; Gille et al., 2018b).

The major ways of *M. bovis* transmission between animals are direct contact (eg. through respiratory secretions) and consumption of infected milk (Maunsell et al., 2011). Introduction into a herd usually happens through purchase of replacement animals (Fox et al., 2005). However, several other ways of *M. bovis* introduction and transmission might have been neglected in the past. Troubling recent illustrations are the introduction of *M. bovis* in two Finnish herds by use of contaminated artificial insemination semen (Haapala et al., 2018), and the first detection of *M. bovis* in New Zealand in July 2017 (McDonald et al., 2009, Ministry for Primary Industries, 2017). In the latter outbreak, import of embryos, feed, fomites, semen and other animal species were investigated as sources of this introduction, since no live cattle were imported since 2013, but to date the source remains unidentified (Ministry for Primary Industries, 2017). In the current mindset of reducing antimicrobial use and improving animal health, it is imperative to prevent *M. bovis* introduction on farms and countries alike.

Colostrum has been mentioned as a possible source of *M. bovis* in the past (Godden et al., 2006, Maunsell et al., 2012), but to the author's knowledge, no systematic studies on the prevalence of *M. bovis* in colostrum are currently available. Despite this lack of information, empirically designed *M. bovis* herd control programs often advocate the removal or (heat-) treatment of the herd's own colostrum as a precaution measure (Maunsell et al., 2011). Withholding colostrum from neonatal calves is not an option, as they depend on colostrum to bridge the period from birth until their own immunity kicks in (Godden, 2008). Purchase of colostrum from other herds holds a risk for other infectious diseases, especially paratuberculosis (*Mycobacterium avium* subsp. *paratuberculosis*) (Streeter et al., 1995) and will not provide the calf with herd-specific maternal immunity. Decontaminated colostrum (pasteurized or gamma irradiated) can be bought, but this will result in a significant financial burden. Heat treatment lacks, especially in smaller farms, economical and practical feasibility due to the small amounts to be processed. Knowledge on the prevalence of *M. bovis* in colostrum is essential to guide

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farmers in the choice of which preventive- or control measures are preferentially taken when considering within- and between herd spread of *M. bovis*. Therefore, the main objective of this study was to determine the presence of *M. bovis* DNA in colostrum from herds with a recent *M. bovis* infection. A secondary objective was to determine if a seasonal effect could be seen in selected herds that were followed over time.

A survey was conducted on seventeen farms throughout Belgium. Farms were conveniently selected by the local veterinarian and samples were collected throughout 2016 and 2017. The inclusion criteria were a recent (< one month) *M. bovis* infection in the herd, documented by either positive culture or PCR, and the willingness of the farmer to participate. Farms could be either beef, dairy or mixed type. Four beef, five dairy and eight mixed farms participated. Sample size calculations were preset on the available budget, which allowed the analysis of up to 370 samples. Based on an average herd size of 80 lactating animals and with an expected prevalence of 25% of the animals shedding, ten animals needed to be sampled in each herd to detect *M. bovis* with 95% confidence. A limit of twelve samples per herd was set on thirteen farms. Four farms were sampled for a longer time (six to twelve months) to determine seasonal variation and to possibly gather better insight on how to interpret the results in other herds. Colostrum samples were collected immediately post-partum after disinfection of the teats with gauze drenched in alcohol. A cow composite sample (pooled sample of all four quarters for each cow) was taken for each cow in a 15 mL Falcon™ tube (Fisher Scientific). Sample collection and subsequent storage at -20°C was performed by the farmer. Farmers were informed on the most ideal sampling procedure, and provided with the necessary material to perform this in a repeatable fashion. The samples in the thirteen herds that had a maximum of twelve samples were analyzed individually at the laboratory of ARSIA for presence of *M. bovis* DNA by real-time PCR (VetMAX™ *M. bovis* kit, ThermoFisher Scientific), targeting the uvrC gene. Before analysis, samples were mixed with PBS, centrifuged and the supernatans discarded. A mix of proteinase K/ATL buffer (Qiagen) was added to the pellet before cell lysis. DNA was automatically extracted by use of the MagAttract 96 cador pathogen kit (Quiagen) and KingFisher™ Flex 96 Deep-Well Magnetic Particle Processor (Thermo Fisher Scientific™). All samples with a Ct below 40 were considered positive.

In the case of the four herds with a longer time of follow-up, testing was performed by the laboratory of the Milk Control Centre (MCC) after pooling the cow composite samples. In the MCC laboratory, pooled samples were examined using the real-time PCR Pathoproof® Complete 16-kit (Thermo Scientific) according to the supplier's manual. Each pool consisted of a maximum of five cow composite samples of cows belonging to the same herd. Because of the consistency of colostrum, one third of the pools for examination were analyzed in duplicate with an adapted protocol (dilution of the colostrum to 1/10 in sterile water and an extra 5 min incubation during the DNA-extraction). There was no difference in results between the two methods and as such the standard protocol was used. Colostrum samples of the *M. bovis* positive pools were examined individually the next day. Ct-values below 37 were considered as positive; Ct-values between 37 and 40 were considered borderline, but positive.

Data were kept in Excel 2016, and analyzed by SPSS version 25 (IBM, Armonk, New York, United States). In total, 368 colostrum samples from seventeen herds were analyzed (table 1). *M. bovis* DNA was detected in 1.9% (7/368) of these samples, positive samples were obtained from four different farms. Thirteen of the 17 sampled farms did not have any *M. bovis* positive colostrum samples. On the four farms that did have positive samples, on-farm/within herd prevalence ranged between 2.8% (2/71) and 30% (3/10). The average within herd prevalence was 3.2% (Standard deviation: 4.9%; Range: 0-30.0%). Of the seven positive samples, four samples yielded a borderline positive Ct value (>37 and <40), probably indicating a low *M. bovis* DNA-content or a false positive result (Table 2).

To the authors knowledge, this is the first study aiming at determining the prevalence of *M. bovis* in colostrum. Because colostrum samples can only be collected at one time point (just after calving), the decision was made to have the sampling performed by the farmer. Unfortunately not all farmers complied 100% with the protocol and did not send in the twelve samples required for each farm to achieve the desired level of precision. Several PCR positive samples had a high Ct value, indicating only a marginal amount of *M. bovis* DNA present in the sample. Very high Ct values may indicate carryover of DNA between samples (Klaas et al., 2016). Even though all farmers were instructed to take milk samples as cleanly and aseptically as possible through an on-site demonstration, it is possible the actual sampling was not done lege artis in every case.

The main finding of this study was that *M. bovis* DNA could be detected in colostrum in a small amount of samples. In the herds where a longitudinal follow-up was done over the year, only two positive samples were found on a total of 258 samples, while *M. bovis* was still circulating in the herd during the entire study (based on sampling of clinical cases). Because of the low amount of positive samples, no conclusions on seasonality could be made based on the results of these herds. The use of PCR methods that were manufactured for the use on milk could also have influenced the diagnostic accuracy on colostrum. With the PCR assays used in this study, *M. bovis* DNA was only sporadically detected in colostrum. It is unclear whether the amount of bacteria present in colostrum would suffice to infect the calf, especially in the case of marginally positive samples. Furthermore, the viability of *M. bovis* in these samples was not determined in this study. Previous research has shown a one log reduction of *M. bovis* CFU/ml when colostrum was frozen and subsequently thawed (Gille et al., 2018a).

Our results seem to indicate that the prevalence of *M. bovis* is that low that prospective economic damage of the within-farm transmission risk doesn't outweigh the economic disadvantages of purchasing colostrum from other herds or investing in pasteurization equipment on already infected farms. This requires, however, confirmation in a larger study in which preferably different techniques are used to demonstrate the presence of *M. bovis* in colostrum, including isolation of the agent.

M. bovis negative herds on the other hand should consider preventive measures such as pasteurization or gamma irradiation when buying in colostrum from other farms, to prevent the introduction of *M. bovis* in the herd. Alternatives such as on farm acidification (Parker et al., 2016) might be an option, but warrant further research, especially concerning the preservation of maternal antibodies in colostrum. Freezing and subsequent thawing was shown to reduce the amount of colony forming units by one log (Gille et al., 2018a), which could mean that, in the case of low level *M. bovis* contamination, this treatment could lessen the risk of infection. However, no infectious dose has been established for *M. bovis* yet, so any future applications should be researched further. Attention should be paid to avoid contamination while sampling, to avoid incorrect decisions to remove animals from the herd. Discarding colostrum of cattle with a known *M. bovis* infection is likely a good advice.

A variation of colostral shedding was seen between the tested herds in this study, which could indicate differences in excretion of *M. bovis*. Hypothetically, this could be based on the time of introduction of *M. bovis* in the herd, where recently infected herds would have a higher amount of shedding, concurrent with the rapid spread of an *M. bovis* strain through a seronegative population (Arcangioli et al., 2008). However, herd 17 was experiencing a large outbreak of *M. bovis* related disease in adult cattle at the time of sampling, after a primary introduction into the herd one month earlier, without any detectable shedding of *M. bovis* in the colostrum tested.

In conclusion, *M. bovis* contamination of colostrum could be evidenced in a small number of animals on some recently infected herds. Further research on the minimum infective dose is needed, in order to be able to estimate the infection risk from infected colostrum. Measures ensuring purchase of negative colostrum are recommended in *M. bovis* negative herds. In positive herds, the within herd transmission due to colostrum is probably low compared to the other ways of transmission and may not outweigh the negative effects of replacing herd specific colostrum in many herds.

FIGURES AND TABLES

Table 1. Prevalence of *Mycoplasma bovis* in freshly calved cattle

Herd	Type (beef, mixed or dairy)	Total number of cattle in the herd	Percentage PCR positive colostrum samples (positive/number sampled)
1	Beef	161	0 (0/6)
2	Beef	139	0 (0/11)
3	Dairy	74	0 (0/8)
4	Dairy	103	0 (0/3)
5	Mixed	209	0 (0/12)
6	Mixed	121	0 (0/5)
7	Mixed	216	30.0 (3/10)
8	Beef	152	10.0 (1/10)
9	Mixed	205	0 (0/10)
10	Mixed	245	0 (0/11)
11	Mixed	316	0 (0/4)
12	Mixed	433	11.1 (1/9)
13	Mixed	282	0 (0/11)
14	Dairy	587	0 (0/63)
15	Dairy	363	2.8 (2/71)
16	Dairy	311	0 (0/74)
17	Beef	241	0 (0/50)

Table 2. Ct-values of positive samples and their interpretation

Source herd	Ct value	Interpretation
Herd 7	37.14	positive
Herd 7	38.87	borderline
Herd 7	38.2	borderline
Herd 8	38.36	borderline
Herd 12	29.14	positive
Herd 15	34.3	positive
Herd 15	39.8	borderline

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CHAPTER 5

A NEW PREDILECTION SITE OF *MYCOPLASMA BOVIS*: POSTSURGICAL SEROMAS IN BEEF CATTLE

A NEW PREDILECTION SITE OF *MYCOPLASMA BOVIS*:

POSTSURGICAL SEROMAS IN BEEF CATTLE

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ABSTRACT

Mycoplasma bovis is a highly contagious bacterium, which predominantly causes chronic pneumonia, otitis and arthritis in calves and mastitis in adult cattle. In humans, Mycoplasma species have been associated with post-surgical infections. The present study aimed to identify the bacteria associated with three outbreaks of infected seromas after caesarian section in Belgian Blue beef cattle. A total of 10 cases occurred in three herds which were in close proximity of each other and shared the same veterinary practice. *M. bovis* could be cultured from seroma fluid in five of the six referred animals, mostly in pure culture and was isolated from multiple chronic sites of infection (arthritis and mastitis) as well. DNA fingerprinting of the isolates targeting two insertion sequence elements suggested spread of *M. bovis* from chronic sites of infection (udder and joints) to the postsurgical seromas. Identical genetic profiles were demonstrated in two animals from two separate farms, suggesting spread between farms. Mortality rate in the referred animals positive for *M. bovis* in a seroma was 80% (4/5), despite intensive treatment. A massive increase in antimicrobial use was observed in every affected farm. These observations demonstrate involvement of mycoplasmas in outbreaks of postsurgical seromas in cattle.

INTRODUCTION

Mycoplasma bovis is a worldwide emerging bacterium in cattle (Nicholas, 2011, Spergser et al., 2013). This pathogen is highly contagious and a single clone can spread rapidly after introduction into a naive herd (Soehnlen et al., 2012, Timsit et al., 2012). *M. bovis* has mainly been associated with chronic unresponsive pneumonia, otitis and (peri)arthritis in calves (Maunsell and Donovan, 2009) and with mastitis, arthritis and pneumonia in adult dairy cattle (Pfützner and Sachse, 1996). Occasionally *M. bovis* has been reported to cause infections of the tendon sheath (Gagea et al., 2006), keratoconjunctivitis (Alberti et al., 2006), meningitis (as a complication of otitis media/interna) (Stipkovits et al., 1993), myocarditis (Maunsell and Donovan, 2009), genital infections (vaginitis, abortion and vesiculitis) (Pfützner and Sachse, 1996) and decubital abscesses over the brisket and joints in calves (Kinde et al., 1993). Especially in chronic stages, the disease is characterized by a poor response to antimicrobial agents which compromises animal welfare, increases antimicrobial use and causes important economic losses such as weight loss, milk drop, loss of carcass quality, increased mortality/culling risk and increased veterinary costs (Maunsell et al., 2011).

Caesarian section (CS) is a common surgical procedure in Belgian Blue (BB) cattle due to the muscular hypertrophy of this breed (Michaux and Hanset, 1986). Several pre-, peri- and postoperative complications of this surgical procedure have been described (Mijten, 1998, Kolkman et al., 2010). In BB cattle wound infection and seroma formation occur in 13% and 1% of CS cases, respectively (Mijten, 1998). A seroma is defined as a sterile fluid accumulation between subcutis, muscle layers and/or the peritoneum and is a well-known complication after surgery, including CS, also in humans (Chelmow et al., 2004). Seromas can become infected either through hematogenous spread, surgical wound infection or iatrogenic by non-sterile punction (Chelmow et al., 2004).

In the current manuscript, strain typing based on insertion sequence profiling was used to get insights in both intra- and inter-animal spreading of *M. bovis* in an epidemic of infected seromas after CS on multiple farms belonging to a single veterinary practice.

MATERIALS AND METHODS

Animals, examination and sampling

All study animals were adult BB cows that recently underwent CS, originating from three different BB beef herds (farm 1, 2 and 3) of the same veterinary practice. All cows had a chronic site of infection (mastitis or arthritis), a non-responsive high fever and lost weight. Transabdominal ultrasound was performed with a 7.5 MHz transducer (MyLab 25 Gold, Esaote Benelux). All animals had seromas intra-abdominally, visualized by ultrasound. Aspirations were performed under ultrasonographic guidance, using a 21 G needle after local desensitization with procaine 4% (Procainii Chloridum 4% + adrenalinum, Kela, Hoogstraten, Belgium) and surgical preparation of the punction site. Samples consisted of seroma fluid (aspirations from live animals or swabs from the lesions taken at necropsy), joint fluid, milk and swabs of various affected organs post mortem.

Figure 1: Fluid accumulation between the greater omentum and peritoneum in a 4 year old cow (cow 4) suffering from multiple *Mycoplasma bovis* infected seromas.

Microbiological identification

All samples (Table 1) were stored at 4°C and plated within 24 hours on Columbia agar plates supplemented with 5% sheep blood and on a modified pleuropneumonia-like organism (PPLO) agar plate (DIFCO™, BD, NJ) containing 832000 IU/l polymyxin, 0.36 g/l ampicillin, 23.1% deactivated horse serum and 6.5% yeast extract. Agar plates were incubated at 35°C in an atmosphere enriched with 5% CO_2. Blood agars and PPLO agars were incubated for 48 hours and 7 days, respectively. Bacterial isolates were subsequently identified at the species level with standard biochemical methods.
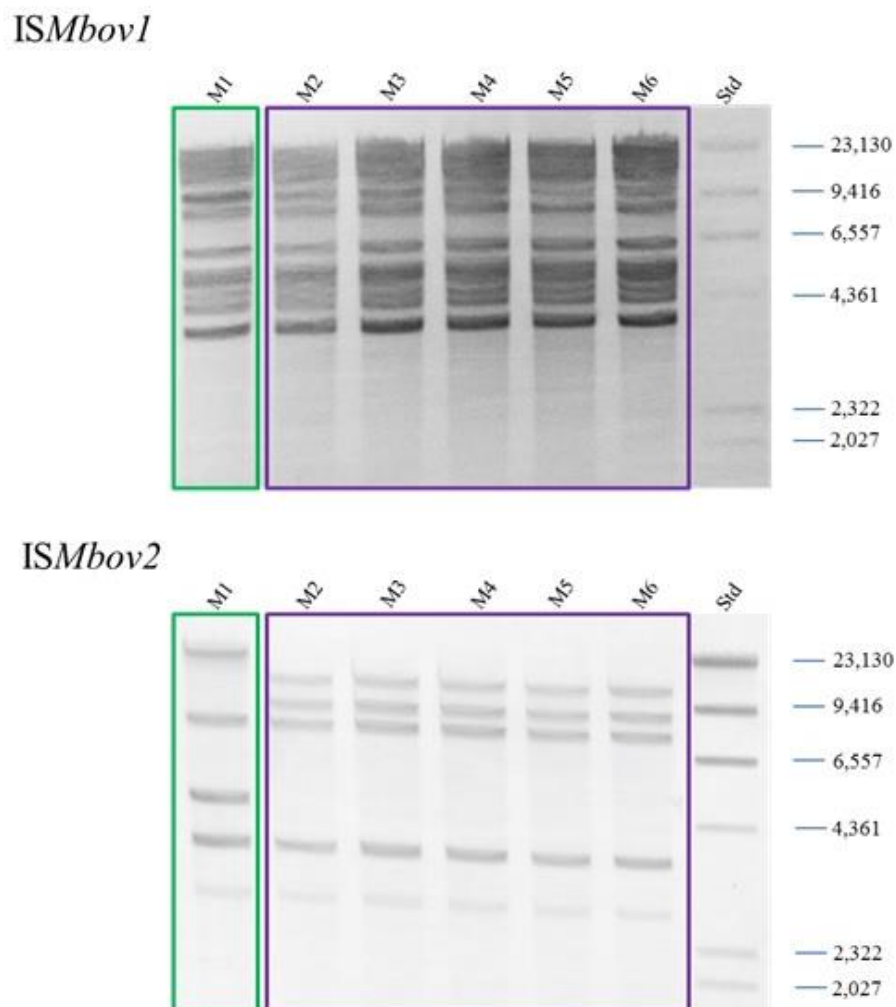
Figure 2: Southern blots of *M. bovis* isolates from cows with postsurgical seroma formation after Insertion Sequence elements typing targeting ISMbov1 and ISMbov2. **M1**: Seroma of cow 3, farm 1, November 2014. **M2**: Seroma of cow 4, farm 1, April 2015. **M3**: Udder cow 4, farm 1, April 2015. **M4**: Udder cow 6, farm 3, June 2015. **M5**: Joint fluid cow 6, farm 3, June 2015. **M6**: Seroma cow 6, farm 3, June 2015. Std: DNA Molecular Weight Marker II, DIG-labeled (Roche)

Presumptive *M. bovis* identification was based on the typical fried-egg colony appearance on modified PPLO agar and the presence of lipase activity as tested on medium containing tween-80 (Devriese and Haesebrouck, 1991).

Species identification and typing by insertion sequence elements typing

After mycoplasmal species confirmation by realtime PCR targeting the *uvrC* gene of filter-cloned isolates (Rossetti et al., 2010), typing was performed by insertion sequence (IS) elements profiling on 6 *M. bovis* isolates (M1-M6) derived from cow 3 (M1, farm 1), cow 4 (M2-M3, farm 1) and cow 6 (M4-M6, farm 3). Cow 5 (farm 2) could not be included because the sample was analyzed by an external lab without preservation of the sample.

DNA extractions from axenic cultures of *M. bovis* were carried out with the peqGOLD Bacterial DNA kit (Axonlab, Baden, Switzerland). IS element typing was performed as previously described (Thomas et al., 2005; Aebi et al., 2012). Briefly, genomic DNA was digested with the *Eco*RV restriction enzyme and DNA fragments were separated on a 0.7% agarose gel and further transferred on positively charged nylon membranes. IS*Mbov1* and IS*Mbov2* IS elements were further detected using digoxigenin-11-dUTP (dig) labelled probes as previously described (Pilo et al., 2003, Aebi et al., 2012).

RESULTS

Clinical evolution

A total of 10 BB cows developed a postsurgical seroma between October 2014 and June 2015 (6 in farm 1, 1 in farm 2 and 3 in farm 3). Of these animals six were available for further examination in this study. The most chronic cases (cow 1 and 2 on farm 1) had been treated with penicillin, neomycin, lincomycin, spectinomycin, amoxicillin, florfenicol and enrofloxacin at the farm without any improvement. They both had a very poor body condition score (1 on a scale of 5) and were immediately euthanized for welfare reasons. Necropsy showed multiple large seromas in the abdomen (Figure 1), abscesses in the subcutis, purulent mastitis on four quarters and arthritis in multiple joints in both animals. The third cow (cow 3) from farm 1 did not suffer from seromas at admission to the clinic, but developed seromas on the left and right side of the abdomen some days after first examination. Blood examination showed a low selenium level (51 µg/l, ref: 70-100 µg/l) and leukocytosis ($46.4*10^9$/l, ref.: $6.0\text{-}9.0 *10^9$/l). The animal was treated with

several antibiotics, clinical response followed when treated with gamithromycin (Zactran®, Merial®, 6mg/kg intramuscular (IM), every 7 days) and neomycin and penicillin (Neopen®, MSD®, 500 mg/100 kg Neomycin, 1.000.000 IU/100 kg penicillin IM, daily) for 5 weeks, together with draining and daily rinsing of the seromas with 0.05% chlorhexidine. The animal was discharged after 35 days. Treatment was continued at home.

At the farm, the local veterinarian preventively medicated every cow with 2.5 mg/kg tulathromycin intramuscularly at the moment of the CS and again 1 week after surgery, in an attempt to stop the development of new cases. In April 2015 a new case (cow 4; BB; 5 years old, 17 days post calving) occurred in the same farm. The animal initially presented fever, which did not respond to treatment with neomycin-penicillin, penicillin, tulathromycin, tylosin and trimethoprim-sulfonamide. The cow developed arthritis on both carpal joints and mastitis with secretion of sandy, granular sediments in all quarters. Ultrasonography confirmed the presence of multiple fluid filled structures bilaterally in the abdomen. Necropsy showed multiple seromas and general lymphadenopathy. On blood examination, serum selenium levels were very low (16 µg/l, ref.: 70-100 µg/l).

In June 2015 two other farms, belonging to the same veterinary practice, were affected by the same condition. Multiple animals were affected, samples were collected from two animals (cow 5 from farm 2 and cow 6 from farm 3) (Table 1).

Bacteriology and typing of isolates

An overview of the samples taken from the 6 cows with post-surgical seroma formation from the three farms and bacteriological culture results are presented in Table 1. *M. bovis* was isolated in pure culture from the post-surgical seromas in 4 out of 5 cases sampled and in one case together with *Trueperella pyogenes*. No seroma fluid was collected from cow 2. In several animals *M. bovis* was also cultured from the udder and/or joints (Table 1).

IS elements typing showed a single *M. bovis* profile in animals from herds 1 and 3 (cows 4 and 6, respectively) (Figure 2). In herd 1 the isolates from cow 3 (November) and cow 4 (April) were very similar, especially when using IS*Mbov1* IS elements, but not identical. A

single IS element profile was observed in the udder and seroma fluid of cow 4 and in the udder, joint and seroma fluid from cow 6 (Figure 2).

Table 1. Bacterial culture results from different sampling sites of 6 beef cows with post-surgical seroma formation.

	Cow 1	*Cow 2*	*Cow 3*	*Cow 4*	*Cow 5*	*Cow 6*
Sampling site						
Joints	*T. pyogenes* and *M. bovis*	*M. bovis*	N/A	N/A	N/A	*T. pyogenes* and *M. bovis*
Udder	*T. pyogenes* and *M. bovis*	*M. bovis*	*M. bovis*	*M. bovis*	N/A	*T. pyogenes* and *M. bovis*
Kidney	N/A	Negative	N/A	N/A	N/A	N/A
Lung	Negative	N/A	N/A	N/A	N/A	Negative
Seroma fluid	*M. bovis; A. fumigatus* (separate seromas)	N/A	*M. bovis*	*M. bovis*	*M. bovis*	*T. pyogenes* and *M. bovis*

***M. bovis: Mycoplasma bovis; T. pyogenes*: *Trueperella pyogenes*; *A. fumigatus*: *Aspergillus fumigatus*, N/A: not available**

Cow 1-4: Farm 1, Cow 5: Farm 2, Cow 6: Farm 3.

DISCUSSION

In human medicine, wound infection caused by *Mycoplasma hominis* is a reported complication of CS (Phillips et al., 1987, Maccato et al., 1990). In the present study, *M. bovis* was isolated from seromas following CS in five cows from three different farms. To the author's knowledge the involvement of *Mycoplasma spp.* in wound infections or seromas in animals has not been previously reported. Possibly the importance of *M. bovis* and other *Mycoplasma spp.* is underestimated, since the selective growth media required for their isolation are rarely used in routine bacteriological examinations of wound infections and *Mycoplasma spp.* either don't grow or are easily overgrown on regular blood agar plates.

In the present outbreaks, *M. bovis* infections resulted in a severe clinical presentation with a very poor outcome. A single animal could be saved after a long hospitalization period. The lack of clinical break point values for *M. bovis* made the selection of an effective antibiotic difficult, resulting in the use of different molecules. However, given the chronic state of disease at the time of submission, little efficacy of antimicrobial therapy is to be suspected and euthanasia is strongly advised for both economic and welfare reasons. Next to the loss of the animals, the condition resulted in a massive increase in antimicrobial use in the affected farms, partly due to therapy failure and partly because of the fear of the development for new cases. Despite the apparent improvement of the situation after preventive antimicrobial use, the authors wish to underscore that no evidence to support this measure is provided. In contrast, such prophylactic treatments might further increase antimicrobial selection pressure, selecting for multi-drug-resistant bacteria, including *M. bovis* isolates as recently reported in France (Gautier-Bouchardon et al., 2014).

M. bovis is known to spread hematogenously from the lungs to the joints (Maunsell and Donovan, 2009). The current case series strongly suggest hematogenous spread of *M. bovis* to the surgical incision site from a primary site of (chronic) infection, most probably the udder and joints. The presence of identical isolates in different body sites of the same animal also points in this direction. It is not clear why this spread to the seroma occurred: both virulence factors related to the strain or physiological immune suppression due to parturition might have played a role.

Possibly, impaired immunity following parturition facilitated dissemination of *M. bovis*. Also the low selenium levels of cow 3 and 4 might have played an additional role in the

illness, as this deficiency has been linked to a lowered periparturient immunity level (Sordillo, 2013).

A most interesting observation was the presence of an identical isolate in two different farms, suggesting the spread from one farm to the other. Direct spread through animal contacts is not likely since there were no connecting pastures or animal transports between the farms. Airborne transmission of *M. bovis* from one herd to another cannot be excluded, but has not yet been demonstrated in cattle (Soehnlen et al., 2012). Therefore, indirect spread through materials or persons, visiting both farms, is the most probable route of transmission.

In conclusion, this report adds infection of post-surgical seromas to the list of *M. bovis* associated pathology. The condition can affect multiple cows in a short period and has a poor prognosis. Early detection by farmers and practitioners of animals with chronic sites of *M. bovis* infection and subsequent culling is likely to be the best option to prevent or contain this pathology. Appropriate biosecurity precautions should be taken by all farm visitors including attending veterinarians to prevent transmission among herds.

ACKNOWLEDGEMENTS

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CHAPTER 6

GENERAL DISCUSSION

Mycoplasma bovis is a pathogen which is seen as one of the rising threats in modern-day cattle farming. Its capacity to hide in apparently healthy animals, combined with its highly infective nature makes it hard to stop its spread once present. As such, the aim of this thesis was to map the presence of this bacterium in Belgian herds, and fill in gaps in the epidemiological knowledge of *M. bovis*, to be able to give the best possible and scientifically underbuilt advice on prevention and control. The most important conclusions from this thesis and their practical implications are discussed below.

PREVALENCE OF *MYCOPLASMA BOVIS* IN BELGIUM (CHAPTER 3)

In a study performed in 2009, 1.5% of all Belgian dairy herds tested positive for *M. bovis* on BTM culture (Passchyn et al., 2012). However, field reports and laboratory analysis in recent years seemed to indicate a rise in *M. bovis* prevalence and subsequent disease in Belgian herds (Unpublished data, DGZ). The development of ELISA and PCR tests for use on BTM samples provided a quick and easy-to use alternative to bacterial culture for herd-screening purposes (Nielsen et al., 2015; Petersen et al., 2016). We used these novel tools to determine the prevalence of *M. bovis* in Belgium, and found it a lot higher than expected. Where repeated culture, which is somewhat comparable to PCR in sensitivity (Gonzalez and Wilson, 2003), only resulted in a 1.5% prevalence (Passchyn et al., 2012), PCR on BTM in this study resulted in 7% of all farms testing positive. Since *M. bovis* excretion is intermittent, this amount of infected farms is possibly even an underestimation: if shedder cows are separated from the milking herd or are dry at the time of testing their milk would, as such, also not be included in the BTM (Gonzalez and Wilson, 2003). The results become even more interesting when looking at the antibody ELISA tests performed on the same samples as the PCR. A true prevalence of 24.8% was found, indicating that one out of four farms in Belgium had contact with *M. bovis* in previous months. This number goes above 30% when PCR results are included, as there was no overlap between PCR and antibody ELISA positive farms in this study.

Several explanations are possible for this non-overlap between the two tests. As shedders can excrete *M. bovis* intermittently, it is possible that part of the persistently infected animals were not shedding at that moment. Animals with clinical mastitis might have been separated from the milking herd as well, resulting in a negative BTM sample on PCR while other animals could still contribute to the antibody level. One could argue that the presence of *M. bovis* could cause the antibodies present in the BTM sample to bind to the

bacteria, instead of to the antigen provided by the ELISA. This could be the explanation why, in a study by Parker et al. (2017), no overlap could be seen between PCR and ELISA analysis on BTM either. Another possible explanation to this discrepancy is that antibodies as measured on BTM in the aforementioned previous study by Parker et al. (2017) dropped below the detection limit at 8-12 months after the first *M. bovis* introduction, whilst *M. bovis* could still be detected by PCR occasionally. One more possibility is that PCR will be positive faster compared to antibody ELISA, and might already test negative again by the time the ELISA tests positive. Recent findings also showed that the commonly used BIO-X K302 has a far lower sensitivity compared to a MilA ELISA, possibly also influencing results (Petersen et al.,2018b).

In any way, the prevalence of *M. bovis* DNA in BTM seems a lot higher in Belgium compared to other recent studies from Denmark, the Netherlands and Southern France (See figure 9 p 27) (Arcangioli et al., 2011; Nielsen et al., 2015; Hogenkamp, 2017). A possible explanation on this discrepancy might be the high density of cattle farms in Belgium and the high level of trade going on between these farms (Ensoy et al., 2014). However, the same high density and complex contact structure is present in the Netherlands, and a recent study only found a BTM prevalence of 1% in this country (van Klink and Koorevaar, 1999; Hogenkamp, 2017). In conclusion, the combination of both Ab ELISA and PCR tests on BTM will result in a higher accuracy and is therefore recommended.

The seroprevalence of *M. bovis* was also determined in Belgian beef herds on samples from the 2014 Belgian winter screening for regulated diseases (Gille et al, unpublished results). In 100 at random selected herds, five serological samples were conveniently taken. Herds were considered positive when at least one sample was positive. The between herd prevalence was 21%, with the within herd prevalence ranging from 20 to 60% (Gille et al, unpublished results). The seroprevalence of *M. bovis* in Europe shows a high variety between countries, ranging from almost nonexistent in the Nordic countries to 100% in Hungary (See figure 9 p 27). Comparison between serology and BTM ELISA results is difficult at this time, as serological research has shown an underdiagnosis of cases if only milk is analyzed, since antibodies will only appear in the presence of *M. bovis* mastitis (Petersen et al., 2018a). As such we are unable to say which sector is most affected by *M. bovis* in Belgium at this time. Both beef and dairy herds are still far less

affected compared to the veal industry, where previous work resulted in isolation and seropositivity in almost every herd (Pardon et al., 2011; Pardon, 2012).

In conclusion, the high prevalence of *M. bovis* in both beef and dairy herds in Belgium in recent years underscores the need for herds currently negative to guard their biosecurity. Testing animals at the time of purchase is crucial. The use of PCR and Ab ELISA in parallel testing on BTM from a prospective herd before purchasing cattle from said herd could mean an improved security to make sure *M. bovis* is not introduced as well. When purchasing non lactating animals or purchasing from a non-dairy herd, serological or swab sampling of the animals and/or a randomized sampling of some animals of the herd is advised. It is unclear whether an animal testing positive on serology is a carrier, and some clinically ill animals might not have detectable antibodies either, but if one or more animals or the BTM test positive, and the prospective herd is negative, no animals should be purchased to avoid the introduction of *M. bovis* in the herd.

RISK FACTORS FOR THE PRESENCE OF *M. BOVIS* IN A HERD

In the second part of the first study of this doctoral thesis (Chapter 3), risk factors for a herd testing *M. bovis* positive on BTM were investigated. Previous studies identified purchase as the main risk factor for *M. bovis* presence on herd level, which seems logical since this is one of the main pathways of introduction into a herd (Gonzalez et al., 1992; Maunsell et al., 2011). However, even though purchase in the year before the positive test was found to be a possible factor on univariable analysis in our study, this factor was linked to the presence of a breeding bull, and of these two the breeding bull was more significant after stepwise backward elimination. This could imply that the role of the bull in *M. bovis'* epidemiology might have been underestimated in the past. In previous studies, the bull might not have been taken into account when building the risk factor analysis model. The use of a teaser bull could not be taken into consideration due to the low amount of farms that used one in this study.

The possible importance of the presence of a bull ties in with the recent findings of Haapala et al. (2018), demonstrating the use of AI semen from infected bulls as the cause of *M. bovis* introduction into previously seronegative herds in Finland. Since farmers grow more and more conscious of biosecurity and closed herds become more common, focusing only on the purchase of live animals as a possible source is likely insufficient. More focus should be placed on other possible ways of *M. bovis* introduction. This is

illustrated by the recent introduction of *M. bovis* into New Zealand (NZ), a country which had not imported any cattle in the last five years before detection and only 110 animals in total in the 5 years before (Ministry for Primary Industries, 2017). In this instance as well, AI was looked at as a possible source, but it was not the only suggested way of introduction. In the last 10 years, an average of around 240000 straws of bovine semen and 565 bovine embryos were imported into NZ every year (Ministry for Primary Industries, 2017). The antimicrobials used as a means of decontamination of AI semen were reported as possibly ineffective for the complete inactivation of *M. bovis* (Bielanski et al., 1989; Visser et al., 1999; Ministry for Primary Industries, 2017). Next to this, other fomites such as imported feed and farm equipment and the import of other ruminant species were considered as well. In this thesis (chapter 5) we illustrated that the veterinarian can act as a possible passive transmitter of the bacteria as well. This was also described by Gonzalez et al. (1992), who saw all herd visitors in contact with manure and bodily secretions of cattle as a transmission risk.

Next to the identification of the bull as a risk factor, the presence of a separate calving pen was found to be a protective factor. A distinction between a single- or group calving pen could not be made due to a lack of power. The protective effect of the calving pen might be linked to another part of *M. bovis*' epidemiology often disregarded in the past, which is the presence of *M. bovis* in vaginal secretions, especially at calving and during abortion (Stalheim and Proctor, 1976; Bocklisch et al., 1986). It is possible that the protective effect stems from the separation of the (stressed) calving animal from the herd. Stress and cortisol were shown to exacerbate disease and induce shedding in infected animals (Caswell and Archambault, 2008; Alabdullah et al., 2017; Alabdullah et al., 2018). Furthermore, the periparturient period in itself gives cause for immunosuppression, possibly giving *M. bovis* another chance to start shedding or cause disease (Sordillo, 2013). When separating the animal around parturition, this will shield the other cattle from a possible high load of *M. bovis* shed during the parturition, either through vaginal secretions or nasal secretions. Furthermore, this might protect the cow herself in the period where she will be most vulnerable immunity wise, possibly avoiding an infection of cows who will be at the peak of their lactation soon and as such production wise will be a bigger contributor to the BTM. In any case, the definite reason of the protective effect of the calving pen is unclear at this time. As such, further research on the presence of *M. bovis* around calving (and survival in the environment) is necessary, especially since the

colostrum study in chapter 4.2 seems to suggest that there is little indication of periparturient rise of *M. bovis*, as only a few colostrum samples contained *M. bovis* DNA.

Interestingly, both the bull and the absence of a calving pen were identified as risk factors in an article by Kemmerling et al. (2009) on the prevalence and risk factors of *Chlamydia* sp*.* in cattle herds. Ruminant *Chlamydia* species such as *Chlamydia abortus* and *Chlamydia pecorum* can also be the cause of pneumonia, arthritis, conjunctivitis and reproductive problems and generally get transmitted via veneral or feco-oral routes (Kemmerling et al., 2009). Kemmerling hypothesized that the relative risk of transmission using AI was limited to those animals for which semen of a certain bull is used, whereas if only one bull is used on a farm, all bred animals are at risk. The same reasoning can probably be used in the relative risk of *M. bovis* semen as well. An added difficulty in the role of the bull as transmitter is that, even when the bull tests negative at purchase, they can get infected when used for breeding in a seropositive herd, without any clinical signs of being infected. Afterwards, this animal could possibly infect a large amount of cows while escaping detection as this is generally done through *e.g.* milk testing (Hazelton et al., 2018a).

On univariable analysis, next to purchase, the use of a breeding bull and the absence of a calving pen, several other factors were identified which could warrant further investigation. In regard to youngstock raising, individual housing of young calves seemed to be protective, but this factor was removed due to too few farms not using this management system (2/2 farms not using individual housing tested positive on BTM). The use of a high-pressure cleaner to clean calf pens between occupation seemed to be a protective factor as well. Since direct contact is an important way of transmitting *M. bovis* in young calves and contact with fomites had been implicated in the past, these risk factors seem logical. However, other prospective risk factors were less logical. The self-reported presence of otitis in a farm was not predictive for having *M. bovis*. In fact, all farms (5/5) reporting to have otitis (interpreted by the presence of head tilt) in youngstock did not test positive on BTM. A possible explanation for this matter could be that the BTM testing positive is in no way linked to circulation of *M. bovis* in youngstock, and could be tied to strain dependent disease presentations (mastitis vs otitis in calves) or another disease causing otitis (such as *H. somni*) being present. Tubed feeding of colostrum was also a rather unexplainable risk factor for *M. bovis*. In this case, it is possible that this is caused by a confounder: the knowing that *M. bovis* is present could have led farmers to take

control measures on the farm, one of which is ensuring a good immunity transfer. Furthermore, this factor also had relatively little responses on one side, which can have influenced results.

In conclusion, the importance of semen and genital secretions in the transmission of *M. bovis* should be reevaluated. Although AI semen has been the cause of introduction in herds before, the amount of animals affected inside a herd is possibly lower compared to the use of a breeding bull. However, the global impact of infected AI semen could be higher, especially if the carrier animal is in high demand. Surveys in AI centers should be considered to map the spread and impact of the bacteria in these facilities, but the choice of technique should be evaluated as well. Recent research by Vähänikkilä et al. (2018) has shown that real time PCR is an excellent method to detect *M. bovis* in semen samples, but as this method only detects *M. bovis* DNA, no distinction can be made between live and killed *M. bovis*. Furthermore, the *M. bovis* concentration in semen and the infective dose are both still unclear. Next to this, the presence of *M. bovis* in vaginal secretions and amniotic fluid and the possible transmission risk should be investigated as well. One recent study by Hazelton et al. (2018b) found that 18.8% of all cows having a clinical *M. bovis* mastitis were also shedding *M. bovis* vaginally. In Flanders, in 2015, in more than 66 % of the investigated abortion cases (n=5293) no cause could be found on aerobic culture (DGZ, unpublished results). Since *M. bovis* was shown to cause abortions in experimental and clinical settings, investigations into the role of *M. bovis* in abortion cases in seropositive herds might be interesting as well (Watson et al., 2012).

MYCOPLASMA BOVIS AND COLOSTRUM

In chapter four of this thesis, we zoomed in on colostrum as a possible source of *M. bovis* infection for neonatal calves. The assumption of its infective nature has been made previously, but no scientific evidence of *M. bovis* even being present in colostrum was provided until now (Godden et al., 2006). As colostrum uptake is an essential part of the immunity in newborn calves, withholding colostrum as a preventive measure will have a large impact on the newborn calf. In farms, the colostrum surplus is often frozen to act as a reserve at a later time if for example another dam does not produce enough colostrum or the colostrum has a low quality. Trade of frozen colostrum between farms is common in Belgium as well, especially from dairy farms, which often have a surplus (DGZ, 2014).

Decontamination of colostrum by use of batch pasteurization or gamma irradiation is possible but costly and hard-to implement in smaller farms. Pasteurization of colostrum is a very temperature sensitive process, as the immunoglobulins present will denature when heated, possibly resulting in a too low immunoglobulin content post pasteurization if performed incorrectly (Godden et al., 2003; Godden et al., 2006; McMartin et al., 2006). Even when performed correctly, a drop in immunoglobulin content is visible, however, when calves are given enough colostrum (4L) in the critical window, no difference is visible between non-pasteurized and pasteurized colostrum feeding (Godden et al., 2003). Recently, small-batch pasteurizers have come on the market as well, enabling farmers to pasteurize two to four liters of colostrum at a time, freezing it and feeding it to the next calf born on the farm. In the first part of this section (chapter 4.1), the effect of freezing colostrum on the survival of a known concentration of *M. bovis* was tested. A single freeze-thaw cycle resulted in a one log drop of the *M. bovis* CFU/ml. A second freeze-thaw cycle resulted in another 0.5 log drop. These findings have shown that freezing is probably not a valid control measure in the decontamination of colostrum from *M. bovis* and that freezing of colostrum samples does not make it unusable for further epidemiological studies or monitoring programs on *M. bovis* through bacteriological culture.

In the second part of this section (Chapter 4.2) the actual presence of *M. bovis* DNA in colostrum was assessed, by use of a realtime PCR. Of a total of 370 samples, only seven samples tested positive for *M. bovis* DNA. Next to the cross-sectional data gathered on thirteen farms, four farms were followed up over a longer time period, in the hope of identifying risk factors for having a positive colostrum sample. Unfortunately, due to the low amount of positive samples, no risk factor analysis could be performed.

However, since *M. bovis* circulation was confirmed on all farms throughout the study and very little colostrum samples tested positive, the importance of colostrum as a carrier for *M. bovis* might be relatively low compared to other agents, especially when colostrum is fed on a 1-on-1 basis. However, since only one farm was confirmed to be in the acute phase of a *M. bovis* outbreak, conclusions need to be interpreted carefully, as significant shedding in the acute period of infection cannot be excluded completely. Of course, PCR is only capable of detecting DNA, not the live bacteria, and therefore further research on this substrate is necessary. Furthermore, the PCR tests used for the assessment of the presence of *M. bovis* DNA in colostrum were not developed for this substrate, possibly

reducing the sensitivity. Interaction of the test with other *Mycoplasmataceae* is always a possibility as well.

Notwithstanding these limitations, the conclusion of this chapter is that, in herds where *M. bovis* circulation is confirmed, colostrum probably carries a relatively low risk of infection compared to other routes. In these herds, focus should lay on the prevention of infection of young stock through more common ways, such as direct contact and the ingestion of infected milk. Pooling of colostrum needs to be avoided. Treatment of colostrum through pasteurization can be done at 60°C for 30 minutes, but care should be taken not to overheat the colostrum (Godden et al, 2003). Other treatment methods such as acidification of colostrum to a pH of 4, which has been used before on *M. bovis* infected milk, with a successful destruction of all *M. bovis* present after 1 hour, might be investigated in the future (Parker et al., 2016). In herds without a known *M. bovis* presence, purchase of untreated colostrum from other farms should be avoided at all cost to negate the transmission risk.

An interesting juxtaposition in the findings of this thesis is the seeming absence of a periparturient rise for *M. bovis* in colostrum in chapter 4.2, which contrast the findings in chapter 3 that indicate that calving cattle could possibly pose a risk for *M. bovis* circulation on the farm, and the findings in chapter 5 that showed that several of the animals affected by *M. bovis* seromas also had a very low selenium level, which can cause a lowered immunity and a higher risk for infection. A possible explanation of this juxtaposition is the fact that in endemic herds only a relatively low percentage of animals will be carrier of *M. bovis* (Timonen et al., 2017), and therefore only a few animals might be experiencing a periparturient rise and shed *M. bovis* in colostrum. Further research on shedding in colostrum on recently infected herds with the use of different techniques, and research of the importance of *M. bovis* shedding in vaginal and periparturient excretions will hopefully aid in providing an explanation of this juxtaposition.

MYCOPLASMA BOVIS AND SEROMAS

In chapter 5, a case series is described of a cluster of postsurgical seromas in beef cattle (Belgian blue) after CS, in combination with high fever and typical *M. bovis* associated diseases such as mastitis and arthritis. Through culture *M. bovis* could be isolated out of 10 cases. After subsequent strain typing, the strain pattern found on two separate farms indicated a spread from one farm to the other. As mentioned above, the veterinarian was seen as a possible link between farms, a feat also described for other bacteria, such as *Actinobacillus lignieresii* (De Kruif et al., 1992; Rossi et al., 2017). In the described case series, one animal was saved after months of treatment. However, the economic relevance and impact on animal welfare of this treatment was questionable as was the resulting high level of antimicrobial use.

Through IS strain typing, the same strain was shown to be present in the seroma, the joints and in milk and udder samples. As such, it was suspected that *M. bovis* spread hematogenous from a primary site of infection to the surgical site. However, this did not explain fluid accumulations of the same nature as the surgical (left sided) seromas on the right side of the abdomen, which were found in some of the affected animals. Furthermore, several animals in this study suffered from a very low selenium level, which could have been a predisposing factor in the severity of the *M. bovis* disease symptoms. Selenium deficiency is a frequent finding in Belgian herds, especially in Belgian Blue beef herds, and can lead to several pathologies, one of which is a suppression of the immunity of the affected animal (Guyot et al., 2009; Mehdi and Dufrasne, 2016).

In the discussion of the paper it was stated that this was the first paper to describe *Mycoplasma sp.* wound infections or seromas in animals. However, a few papers on wound infections have been published before (Ayling et al., 2011)

In response to this paper, the impression of field veterinarians in Belgium was that all postsurgical seromas were caused by *M. bovis*. However, the authors wish to underscore that this was not the conclusion of the above article. In the above article, *M. bovis* was present, and probably did cause seromas in the affected cattle. However, all investigated animals had other lesions where *M. bovis* was isolated out of as well, which could have been the primary infection site from which the bacteria spread to the operation wound. Subsequent research performed by Evrard et al. (2017) showed that only in a minor subset (4.1%) of all examined seroma cases, *M. bovis* could be isolated from the seroma

fluid. Nonetheless, when a seroma (typically diagnosed with ultrasound after the typical symptoms of weight loss and a high fever resistant to therapy appear after CS) is diagnosed together with other *M. bovis* associated diseases such as mastitis or arthritis, care should be taken to avoid the infection of other animals on the farm, especially when the choice is made to try to treat it. Generally, the seromas need to be opened and the seroma fluid drained, which could pose a large infection risk for the rest of the herd, especially since this is often done in the same (often the only) restraining box used for CS, and the seromas can be very large, sometimes containing more than 40 liters of fluid which will splash up when drained.

FUTURE PROSPECTS

The identification of possible new transmission routes (by epidemiological studies, strain typing, infection tests or otherwise) is a good start to develop more effective prevention and control measures. It is important to realize that the infective capacity of the genital pathway, seroma fluid and colostrum still need to be evaluated further, as well as other possible infection routes such as the environment or other animal species. Vaccination might be a way to stop further spread, but uninfected herds especially need easy to use rapid and reliable methods of screening purchased cattle to reduce the risk of introducing the bacteria. A more reliable method of diagnosing persistently infected shedders and advice on how to deal with the disease once introduced is necessary as well. Strain analysis should be done to find out whether *M. bovis* has strain dependent disease expressions (eg. pneumonia; mastitis; otitis;…) as suggested before.

Antimicrobial resistance is a huge problem in human and animal medicine. *M. bovis* is an important driver of (repeated) and unsuccessful antimicrobial use, resulting in a major selection pressure for many other bacterial species. Rapid detection of *M. bovis* is necessary to adapt treatment and prevent the unnecessary use of antimicrobials *M. bovis* is naturally resistant to. Next to fast diagnosis, clinical breakpoints to define antimicrobial susceptibility are urgently needed as well to limit therapy failure and useless antimicrobial use. As such, the availability of a fast, reproducible method for the determination of MIC values is also essential.

The author would recommend (inter)national monitoring of *M. bovis* prevalence and the introduction of farm certifications in European herds to contain further spread. Possibly the information provided in this thesis will help to convince farmers, veterinarians and governmental organizations of the importance of *M. bovis* and the need of a combined effort to mitigate further spread and losses.

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SUMMARY

In the last decade, *Mycoplasma bovis* has evolved to an economically important cause of disease, reduced welfare and antimicrobial use in cattle worldwide. Most commonly known as a cause of mastitis, pneumonia and arthritis, *M. bovis* has been associated with several other clinical manifestations as well. Due to its innate resistance to antimicrobials targeting the cell wall and chronicity linked to several virulence factors such as the immune evasion by Vsps, its capability to produce a biofilm and its ability to hide intracellularly, treatment is often disappointing. In recent years, a decreasing susceptibility to other antimicrobial agents has been reported, with country specific differences.

To date both effective treatment and vaccines are not available. As such, development of prevention and control measures based on identification of risk factors for *M. bovis* spread and disease is seen as the most important way to tackle problems caused by this bacterium. The development of prevention programs aiming to stop the introduction of *M. bovis* onto novel farms, and control programs interrupting the transmission within an already infected farm depends on knowledge of these risk factors and possible infection routes. Where the main transmission routes such as direct contact, milking and the consumption of infected milk are well known, others have been less explored. As such, the general aim of this thesis was to identify previously underestimated infection sources, disease presentations and risk factors, and give an update on the prevalence of *M. bovis* in Belgium.

In the first study of this thesis (Chapter 3), the prevalence of *M. bovis* in Belgian dairy herds was determined by antibody ELISA and PCR analysis of bulk tank milk (BTM). Of all farms, 7.1 % had detectable presence of *M. bovis* DNA in the BTM. On antibody ELISA, the true prevalence was 24.9%. Interestingly, there was no overlap of ELISA and PCR positive herds, leading to a total prevalence of 32% of all Belgian herds being in recent contact with *M. bovis*. This is worrisomely high compared to a previous culture based study in Flanders and to the prevalence levels in neighboring countries. The potential economic damage of this risen prevalence is high, given *M. bovis*' role in cattle disease and antimicrobial use.

Furthermore, in this study two new risk factors for the presence of *M. bovis* in BTM were found: the presence of a breeding bull on the farm and the absence of a calving pen. The use of a breeding bull next to artificial insemination (AI), instead of only using AI, gave the

farms 4.7 times higher odds for having a positive BTM sample. Farms who did not use a calving pen to separate cows at the time of calving had 3.7 times higher odds of testing positive. The identification of these risk factors together with the recent identification of AI as a source of infection might mean the start of more specific searches on the infective capacity of *M. bovis* in the genital tract.

Colostrum is often considered a possible source of *M. bovis* infection. In Chapter 4 the presence of *M. bovis* in colostrum was assessed through two separate studies. In the first study (Chapter 4.1), the survival of *M. bovis* in colostrum through freeze thaw cycles was assessed under different thawing conditions. Freezing and subsequent thawing was shown to reduce the amount of CFU with 1 log after a single cycle, and with 1.5 log after two cycles, with no added effect from the thawing temperature. As such, freezing is probably not a hurdle for epidemiological research where colostrum needs to be frozen before analysis, but relevance as a preventive measure is unclear as long as the minimum infective dose is not known.

In Chapter 4.2, the presence of *M. bovis* in colostrum was assessed by PCR analysis on 368 samples taken on 17 farms. Of these, only 7 samples tested positive, which corresponds with a prevalence of 1.9%. As such, the general assumption that colostrum is a highly infected substance seems to be grossly overstated, and the current control measure of withholding colostrum on already positive farms to prevent spread to neonatal calves might need to be reconsidered. Likely, individual housing combined with the feeding of pasteurized milk or milk replacer is a more effective control in infected herds. However, for *M. bovis* negative herds, purchase of non-decontaminated colostrum should be seen as an infection risk.

The next chapter (Chapter 5) describes the isolation of *M. bovis* out of seromas, a novel predilection site especially important in Belgium because of the high number of caesarean sections performed in the Belgian Blue breed. Ten animals were shown to be affected by postsurgical seromas infected with *M. bovis*. IS typing revealed that the strain isolated from the seromas and other *M. bovis* predilection sites such as the joints and the udder was the same, which could indicate a spread from this primary infection site to the seroma. Through strain typing, spread of the same strain between two farms was shown, possibly linked to the veterinarian.

In conclusion, *M. bovis* is present in a significant number of Belgian herds. Even though purchase of carrier animals is the main cause of introduction of *M. bovis* into new herds, the identification of the bull as a risk factor for a positive BTM and the possible spread of the seroma-inducing *M. bovis* strain by the veterinarian should urge us to investigate other possible causes of transmission as well. Colostrum seems to be of relatively low importance in the transmission in a herd compared to other factors, given the low PCR prevalence, but *M. bovis* DNA can be present. The results of this thesis also point toward the possible importance of both the bull and the exposure of cattle to *M. bovis* at the time of calving (through uterine secretions or immunosuppression) for the within-herd transmission of *M. bovis*.

These additions to the epidemiology of *M. bovis* might aid veterinarians and farmers to develop more effective prevention and control programs in the near future to tackle the spread of this devastating bacterium.

SAMENVATTING

Mycoplasma bovis is in het laatste decennium wereldwijd uitgegroeid tot een economisch belangrijke oorzaak van ziekte, verminderd welzijn en antibioticagebruik bij runderen. Meest berucht als veroorzaker van mastitis, pneumonie en artritis, heeft *M. bovis* daarnaast ook nog verschillende andere ziektebeelden. Behandeling is vaak zeer teleurstellend door de natuurlijke resistentie van de kiem tegenover antibiotica die de celwand als doel hebben en het vaak chronische ziektebeeld gelinkt aan verschillende virulentiefactoren.

Op dit moment zijn noch een bevredigende behandeling noch efficiënte vaccins voorhanden. De ontwikkeling van preventie- en controlemaatregelen op basis van de identificatie van risicofactoren voor de verspreiding van- en het veroorzaken van ziekte door *M. bovis* wordt beschouwd als de belangrijkste maatregel. Preventieprogramma's die de introductie van *M. bovis* op nieuwe bedrijven willen stoppen en controleprogramma's die de transmissie van *M. bovis* in reeds besmette bedrijven willen inperken hebben nood aan deze risicofactoren en identificatie van mogelijke introductieroutes om gepaste richtlijnen te kunnen opstellen. De belangrijkste transmissieroutes, zijn direct contact, het melkproces en de consumptie van geïnfecteerde melk. Andere routes zijn veel minder onderzocht. Het hoofddoel van deze thesis was dan ook kennis toe te voegen aan de reeds bestaande epidemiologische kennis van *M. bovis*, ter bevordering van de ontwikkeling van betere controle en preventie.

In de eerste studie van deze thesis (Hoofdstuk 3) werd de prevalentie van *M. bovis* op Belgische melkveebedrijven bepaald met behulp van PCR en ELISA analyse op tank melk (TM). Wanneer PCR gebruikt werd testte 7.1% van alle bedrijven positief op TM. Met ELISA werd de ware prevalentie vastgelegd op 24.9%. Er was geen overlap tussen de PCR en ELISA positieve bedrijven, wat maakt dat 32% van de Belgische bedrijven in contact was met *M. bovis* op het moment van het onderzoek. Dit gehalte lijkt onrustwekkend hoog in vergelijking met de in 2009 bepaalde prevalentie van 1.5% op cultuur., en ligt ook hoger dan deze bepaald in buurlanden. Deze stijging is -gezien de economische impact van *M. bovis* en zijn rol in verschillende ziektevormen en antibioticagebruik- slecht nieuws. Verder werden er in deze studie twee nieuwe risicofactoren geïdentificeerd voor de aanwezigheid van *M. bovis* in de kudde: de aanwezigheid van een dekstier, en de afwezigheid van een afkalfstal. Wanneer een stier gebruikt werd (eventueel naast kunstmatige inseminatie) bleek het bedrijf 4.7 maal hogere *odds* te hebben op een positief

TM staal. Bedrijven die geen afkalfstal gebruikten (zonder onderscheid of er individueel gestald werd of niet) hadden 3.7 maal hogere *odds* op het positief testen van de TM. De identificatie van deze risicofactoren naast de recente identificatie van kunstmatige inseminatie als infectiebron wijst op het nut van meer specifiek onderzoek naar de infectieuze capaciteit van *M. bovis* in het genitale apparaat.

Colostrum is veelvuldig vermeld als een mogelijke bron van *M. bovis,* echter zonder onderbouwing. In hoofdstuk 4 werd de mogelijke rol van colostrum onderzocht met behulp van twee verschillende studies. In de eerste studie (Hoofdstuk 4.1.) werd het overleven van *M. bovis* in colostrum doorheen verschillende vriesdooicycli geëvalueerd. Het vriezen en vervolgens ontdooien resulteerde in een daling van de hoeveelheid kolonie vormende eenheden met 1 log na een enkele cyclus, en met 1.5 log na twee vriesdooicycli, zonder dat er een invloed van de dooitemperatuur kon vastgesteld worden. Vriezen is aldus volgens dit onderzoek geen belemmering voor epidemiologisch onderzoek waarbij colostrum bevroren dient te worden voor analyse, maar de verdere relevantie als een mogelijke preventieve maatregel is nog onduidelijk.

In Hoofdstuk 4.2. werd de aanwezigheid van *M. bovis* DNA in colostrum onderzocht door gebruik van PCR op 368 stalen afkomstig van 17 bedrijven. Van deze stalen gaven slechts 7 stalen een positief signaal, corresponderend met een prevalentie van 1.9%. De algemene aanname dat colostrum een hoge graad van infectie heeft lijkt dus op basis van onze studie een overschatting. De huidige preventiemaatregelen waarbij geadviseerd wordt colostrum te weerhouden op reeds besmette bedrijven om de spreiding naar neonatale dieren te vermijden moeten dan ook heroverwogen worden ten voordele van andere maatregelen zoals individuele opfok. Voor *M. bovis* negatieve bedrijven echter is de aankoop van niet steriele biest contra-geïndiceerd en moet dit worden beschouwd als een infectierisico.

Het volgende hoofdstuk (Hoofdstuk 5) beschrijft de isolatie van *M. bovis* uit seromas, een nieuwe predilectieplaats die voornamelijk in België van belang is. De aanwezigheid van een groot aantal dieren van het Belgisch Wit-Blauwe ras maakt dat de hoeveelheid keizersneden (en bijbehorende complicaties) in belangrijke mate hoger ligt dan in omliggende landen. Tien verschillende dieren waren aangetast met seromas na keizersnede waaruit *M. bovis* werd geïsoleerd. Insertie sequentie typering wees aan dat de stam die werd geïsoleerd uit de seromas dezelfde was als deze die werd geïsoleerd uit

andere predilectieplaatsen zoals de uier en de gewrichten, wat kan wijzen op een spreiding van de kiem van deze primaire sites naar het seroma. Dankzij stamtypering werd er ook een verband gezien tussen de overdracht van eenzelfde stam tussen twee bedrijven, en de mogelijke betrokkenheid van de dierenarts in deze verspreiding.

Uit de studies vervat in deze thesis kan worden besloten dat *M. bovis* aanwezig is in een significant deel van de Belgische rundveebedrijven. Hoewel aankoop van dragerdieren de belangrijkste oorzaak van introductie is op nieuw geïnfecteerde bedrijven, moet de identificatie van de stier als een risicofactor voor het positief testen van TM en de identificatie van de dierenarts als mogelijke overdrager van de seroma vormende *M. bovis* stam ons aanmoedigen ook andere mogelijke oorzaken van spreiding te onderzoeken. Colostrum lijkt op basis van onze studie van minder belang te zijn in de overdracht van *M. bovis* binnen een reeds geïnfecteerde kudde, maar *M. bovis* DNA kan wel aanwezig zijn. De overdracht van *M. bovis* binnen een kudde door de stier of door de blootstelling van vee aan *M. bovis* rond het kalven (via uteriene secreties of tgv. immunosupressie) kunnen beide een mogelijke rol spelen in de epidemiologie van *M. bovis*, maar verder onderzoek om deze risicofactoren beter te begrijpen is noodzakelijk.

Deze aanvullingen tot de epidemiologie van *M. bovis* kunnen dierenartsen en veehouders hopelijk helpen met het ontwikkelen van meer efficiënte preventie- en controleprogramma's in de nabije toekomst, om de spreiding van deze kiem af te remmen.

CURRICULUM VITAE

Linde Gille werd geboren op 10 oktober 1990 te Leuven. Na het beëindigen van het secundair onderwijs aan het Sint-Albertuscollege in Haasrode, richting Moderne Talen-Wetenschappen, startte ze in 2008 met de studies diergeneeskunde aan de Universiteit Gent. Ze behaalde in 2014 het diploma van dierenarts (optie herkauwers) met onderscheiding.

Onmiddellijk na afstuderen trad ze in dienst van de vakgroep inwendige ziekten van de grote huisdieren (UGent) als doctoraatsstudent, onder begeleiding van Prof. dr. P. Deprez en Dr. B. Pardon en in samenwerking met de vakgroep pathologie, bacteriologie en pluimveeziekten o.l.v. Prof. dr. F. Haesebrouck en Dr. F. Boyen. Zij legde zich toe op de inwendige ziekten en gezondheidszorg van herkauwers en stond, naast dienstverlening voor de kliniek, mee in voor het klinische onderwijs aan de masterstudenten. Zij nam eveneens deel aan de nacht- en weekenddiensten van de vakgroep. Daarnaast was zij betrokken bij diverse 'veepeiler' projecten van Dierengezondheidszorg Vlaanderen inzake het *Mycoplasma bovis* probleem in Vlaanderen.

Linde Gille is auteur of medeauteur van meerdere publicaties in nationale en internationale wetenschappelijke tijdschriften en vakliteratuur en was meermaals spreker op (inter)nationale congressen.

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HANNIBAL

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