Mastitis Pathogens Revisited: *Mycoplasma* spp.

Bart Pardon
Department of Large Animal Internal Medicine, Ghent University
Merelbeke, Belgium

**Introduction**

Different Mycoplasma species, like *Mycoplasma alkalescens* (17%), *Mycoplasma bovigenitalium* (10%), *Mycoplasma canadense* (10%), *Mycoplasma californicum* (3%) and *Mycoplasma bovirhinis* (1%) can be cultured from milk samples, but their clinical relevance is not always clear (Fox, 2012, Gioia et al., 2016). In contrast, the role of *Mycoplasma bovis* (*M. bovis*) as the most frequent (78%) and most pathogenic Mycoplasma species affecting the udder is internationally well recognized (Fox, 2012, Gioia et al., 2016). In Europe the relative contribution of *M. bovis* is even larger, and other species, like *M. bovigenitalicum* and *M. alkalescens* are rarely detected. The reader will likely know and fear *M. bovis* as being responsible for occasional disastrous outbreaks of unresponsive mastitis, although this clinical mastitis is only the tip of the iceberg. For example, in the North of Belgium 5-10 clinical mastitis outbreaks associated with *M. bovis* occur on an annual basis, which represents 0.2% of the dairy farms in this region (MCC-Vlaanderen). In the same region, 7.1% and 24.8% of a random sample of bulk tank milk (BTM) samples was PCR or antibody-ELISA positive, respectively. However, the highest impact of *M. bovis* lies in calf rearing, where the pathogen causes substantial economic losses and creates an important welfare issue by inducing chronic unresponsive pneumonia, otitis and arthritis (Maunsell et al., 2011). The chronicity of *M. bovis* is renowned, but its contribution to acute bovine respiratory disease (BRD) is even larger, being involved in about 30% of natural BRD outbreaks in dairy calves in wintertime (Pardon et al., 2020). In production systems relying on animal purchase like the veal calf or feedlot industry, *M. bovis* prevalence touches nearly 100% (Caswell et al., 2010, Pardon et al., 2011).

The biggest difference between controlling *M. bovis* and other major udder pathogens, is that focusing on the classic advices for contagious mastitis from the 10-point mastitis control program alone is insufficient. The reasons are first that the primary site of infection is the respiratory tract. Second, calves and young stock can be both source of primary introduction in the herd as form an on-farm reservoir (Maunsell et al., 2011). Other major difficulties in controlling *M. bovis* for practitioners are culling decisions, purchase control and monitoring, which are seriously hampered by diagnostic limitations and gaps in epidemiological knowledge.

In this proceedings paper, an update on diagnostic possibilities and limitations for *M. bovis* is given and how this might translate in more effective control and prevention, especially when calves and young stock are included in a more farm-wide approach.

**Epidemiology and Sources of Infection**

For more than a decade *M. bovis* is depicted as an emerging pathogen (Calcutt et al., 2018). Whether this status truly represents massive spread or is due to substantially larger diagnostic efforts in recent years is unclear. Not only have improved insights in the pathogenic role of *M. bovis* awakened interest, also better diagnostic tools have become more widely available at more affordable prices. Different diagnostic tests and samples have been used in different countries, which makes it difficult to provide accurate prevalence estimates for the whole of
the European Union (E.U.). The big lines are that virtually all E.U. countries are infected, with a much higher prevalence in central and Southern Europe compared to the Scandinavian countries (Gille, 2018). The recent introduction of *M. bovis* in New-Zealand and in particular the enormous efforts made by the government in an attempt to eradicate the disease reached international media, especially because of the culling policy. This event underscored the question as to what are the potential sources of *M. bovis* infection. No doubt that purchase of recently infected (sub)clinical animals or more chronic ((sub)clinical) carriers is the most frequent cause of introduction in a naïve herd. To date, the introduction in New-Zealand has not been traced back to an imported animal. In Finland, the use of contaminated semen during artificial insemination (AI) led to two mastitis outbreaks (Haapala et al., 2018). Milk is an important source of *M. bovis*, but likely more crucial in within farm spread than in between farm spread, given that purchase of milk to feed calves is rare (Maunsell et al., 2012). Also, colostrum can harbor *M. bovis*, albeit at low frequency (1.9% of the dams, only in 4 out of 17 tested farms) (Gille, 2018). The importance of airborne transmission is well recognized for *Mycoplasma hyopneumoniae* in pigs, but insufficiently evidenced to play a major role for *M. bovis* (Soehnlen et al., 2012). The bacteria can survive in milk at 4°C for 54 days (Pfutzner, 1984), in straw and sand at 20°C for 10 days and 8 months, respectively (Pfutzner, 1984, Justice-Allen et al., 2010). The importance of persistence of *M. bovis* in the environment for novel or reinfection in a herd is unclear. Transfer through fomites or persons is also more important than previously believed, and especially veterinarians play a role. Not only can the bacteria be cultured from treatment materials (Gonzalez et al., 1992), a recent study showed that a single strain was involved in outbreaks of postsurgical seroma’s on different farms, where the veterinarian was the only visitor (Gille et al., 2016). A recent space-time analysis in Denmark showed that *M. bovis* clusters are short-lived, but high-risk geographical areas could be identified (Arede et al., 2016). Similar observations were made in Canada, using multi-locus sequence typing (Bell-Rogers et al., 2018). In feedlots, clonal spread of a single *M. bovis* strain has been demonstrated, but later in the production cycle, in the chronic stage of the infection, multiple genotypes were found (Castillo-Alcala et al., 2012, Timsit et al., 2012).

In a recent Finnish study, the infection dynamics of the introduction of *M. bovis* in naïve herds were nicely characterized. In 89.5% (17/19) of the infected herds a few cases of clinical mastitis occurred, mostly within 8 weeks after the index case (Vahanikkila et al., 2019). In 26.3% of the farms, positive PCR results on BTM were found, but only in the first month after the index case (Vahanikkila et al., 2019). In 88.2% of these herds, *M. bovis* could be isolated from nasopharyngeal swabs from calves, and half a year later in 57.8% of these herds, calves were still positive. One and 1.5 year after the index case, *M. bovis* was still present in the calves on 47.4% and 31.6% of these herds, respectively (Vahanikkila et al., 2019). In the individual animal, *M. bovis* specific antibodies are detected most frequently from 2-3 weeks after infection on (Wawegama et al., 2014). Within a month after a clinical *M. bovis* mastitis case, using the MilA ELISA, all cows and all youngstock are serum antibody positive, as previously seen in veal calf operations (Radaelli et al., 2008, Vahanikkila et al., 2019). The appearance of antibodies in BTM follows serum antibody production (Vahanikkila et al., 2019). Depending on the ELISA used, antibodies can persist from 6 months to several years, potentially livelone (Vahanikkila et al., 2019).

### Diagnostic Possibilities and Limitations

There are largely three categories of tests for *M. bovis* available, namely microbial culture, PCR and antibody-ELISA (Parker et al., 2018). In addition, MALDI-TOF is a promising technique to fasten diagnosis, both in conjunction with culture and as a single diagnostic test. Efforts to make strain typing more accessible and affordable are ongoing and the possibilities
of whole genome sequencing are fully being explored (Parker et al., 2018). By track and tracing strains, these techniques can aid in the development of better control and prevention. However, to date their use is limited to research or national surveillance settings, rather than applied to the individual farm. Focus is on the techniques that are currently available in practice.

**Microbial culture**
Mycoplasma require specific media to grow (Parker et al., 2018). Most commonly this is done on liquid broth, but also on solid agar *M. bovis* can be cultured. The limit of detection of culture is 100-272 cfu/mL in milk (Sachse et al., 2010). Advantages are an easier clinical interpretation (viable organisms), possibility for antimicrobial susceptibility testing, simultaneous detection of other Mycoplasma species and low operating costs. Disadvantages are the long turnaround time before results are obtained (5-10 days), potential overgrowth by other bacteria (sterile sampling required) and requirement of viable organisms. Most culture systems require PCR or 16S rRNA sequencing to identify the final species, which is expensive and requires additional time. *M. bovis* can also be biochemically identified by lipase activity (Tween80 containing medium) (Van Driessche et al., 2017). This method is not frequently used, but was 70.5% sensitive and 93.9% specific in a Bayesian latent class comparison with real-time PCR (Bokma et al., 2019). At herd level, a single BTM culture was 33-59% sensitive for the detection of *M. bovis* infected herds, when at least one cow in the herd was positive (Gonzalez and Wilson, 2002).

**PCR**
Both conventional and real-time PCR are available for *M. bovis* detection, with the latter being currently more frequently used (both 16S rRNA gene and *uvrC* gene). Detection limits are as low as 2.4 x 10² cfu/mL in milk and respiratory tract samples (Parker et al., 2018), but are usually better in milk, followed by semen and nasopharyngeal swabs (Parker et al., 2017b). When multiple Mycoplasma species are present the *M. bovis* limit of detection increases (Parker et al., 2017b). Advantages are that the organism does not need to be viable, no further identification steps are required, diagnostic sensitivity is very high and a diagnosis can be reached more rapid (several hours- a day). Disadvantages are the higher costs, other Mycoplasma species not detected, and difficulties with clinical interpretation (e.g. detection of non-viable organisms). Sensitivity of PCR on broncho-alveolar lavage samples was 94.8% and specificity 88.9% in a latent class comparison (Bokma et al., 2019). Despite the multiplicity of PCR methods for *M. bovis* used in different laboratories, an interlaboratory evaluation showed comparable performance (Wisselink et al., 2019). PCR is frequently used after microbial culture to confirm *M. bovis*. This serial testing would improve specificity, not sensitivity. To maximize sensitivity, the most sensitive technique, in this case PCR, needs to be the first test. A recent study exploring a high throughput direct-culture-qPCR approach showed a substantial reduction in labor and associated costs compared to the standard approach of first culturing and subsequently PCR (Andres-Lasheras et al., 2019). The PathoProof assays (Thermo Fischer Scientific, Victoria, Australia) are among the most frequently used PCR kits in the E.U., and at herd level they are 97-100% specific for *M. bovis* (Penry et al., 2014). Surprisingly, there are no studies evaluating diagnostic sensitivity and specificity of PCR on milk available, and also the product leaflets of most PCR kits do not report these crucial parameters.

**MALDI-TOF**
In recent years MALDI-TOF MS (matrix-assisted laser desorption-ionization- time of flight mass spectrometry) revolutionized routine diagnostics. The technique is widely used for
identification of bacteria after culturing, including Mycoplasma species (Spergser et al., 2019). Advantages compared to PCR are the much lower costs of analysis. Disadvantages are the poor results with the direct transfer/toothpick method, when starting from solid media. Also, despite having a rapid identification, culture, requiring 5-10 days, is still needed. To overcome this, recently a protocol was developed to directly detect *M. bovis* in bronchoalveolar lavage samples. The technique was 86.6% sensitive and 86.4% specific, and turnaround time was reduced from over 5 days to less than 3 (Bokma et al., 2019). Given the importance of rapid diagnostics to control and prevent *M. bovis*, 3 days is a much more workable timeframe.

**Antibody ELISA**

Next to detecting the pathogen itself, also antibodies can be detected by serum or milk ELISA. Advantages of antibodies are that the animal does not need to be shedding the organism at the time of sampling and that longevity of antibody expression can last for several months. Disadvantages are the lag between infection and seroconversion (2-3 weeks later), uncertainty around cross-reactivity with other organisms and difficulties in interpretation given the substantial variation between different ELISA’s and sometimes poor sensitivity (Parker et al., 2018). The presence of *M. bovis* antibodies does not necessarily mean that the animal is harboring or shedding the pathogen. Different tests are internationally commercialized like the BIO-K260 and BIO-K302 (Bio-X diagnostics, Jemelle, Belgium), Bovicheck *M. bovis* antibody ELISA (Biovet Inc., Quebec, Canada) and ID Screen ELISA (IDvet, Grabels, France). Also, some in-house ELISAs, like the mycoplasma immunogenic lipase A (MiLA) ELISA are used (Wawegama et al., 2014). Comparative studies are important to estimate the true diagnostic accuracy of such tests. On serum samples, sensitivity and specificity of the BIO-K260 were 28% and 100%, respectively and BIO-K302 was 47% sensitive and 96% specific (Schibrowski et al., 2018a). The result for the BIO-K302 were confirmed in a multi-laboratory Bayesian latent class analysis, taking into account the absence of a gold standard reference test (sensitivity= 49.1%; specificity= 89.6%) (Andersson et al., 2019). The ID Screen ELISA performed better, being 93.5% sensitive and 98.6% specific (Andersson et al., 2019). In *M. bovis* infected calves younger than 3 months old, the BIO-K302 response remained below the recommended cut-off, whereas the MiLA ELISA did detect antibodies (Petersen et al., 2018b). Similarly, using the BIO-K260 ELISA in a follow up of natural *M. bovis* outbreaks, only the MiLA ELISA results followed a similar pattern as culture and PCR in youngstock only (Vahanikkila et al., 2019). *M. bovis* antibody titers are lower in milk compared to sera and longevity is shorter. IgG, which is the target of the ELISA, is produced in blood and transferred to milk. Several factors like lactation stage, clinical history of *M. bovis* infection or presence of udder inflammation affect the presence of antibodies in milk (Parker et al., 2018). In one study antibodies in milk were only increased in cows with *M. bovis* mastitis (Petersen et al., 2018a). When applied on BTM, the BIO-K302 ELISA had at the recommended cut-off of the manufacturer (37% optical density coefficient (ODC)) a sensitivity and specificity of 60.4% and 97.3%, respectively (Bayesian model) (Nielsen et al., 2015). At 50% ODC, sensitivity and specificity increased to 43.5% and 99.6%, minimizing false positive results.

Diagnostic sensitivity and specificity vary substantially between the different available ELISA’s, and this has great influence on interpretation of monitoring and purchase control. More information is provided in the next paragraphs.
Control
Control stands for all measures taken to limit spread and consequences of an infection already present in a herd. Several points of the 10-point-NMC program are of crucial importance to control a contagious udder pathogen as M. bovis. However, additional measures are required to better control the infection at herd level.

Milking technique, dry cow therapy and successful treatment
For every contagious mastitis pathogen, an excellent milking technique is essential, and also for M. bovis this is likely the case. However, in recently infected herds with clinical M. bovis mastitis, the use of individual cloths, disinfection udder wash, disposable gloves, post dipping or backflush were all not associated with recovery speed (Punyapornwthaya et al., 2012). A single study cannot drive any firm, general recommendations, but potentially the importance of hematogenous spread to the udder overrules teat-to-teat spread. Milking suspected animals last or using a separate milking machine are logical measures, but actually putting clinically affected animals immediately in complete quarantine, avoiding contact with animals with non-Mycoplasma related diseases in the hospital pen, is likely more efficient.

Dry cow management might be more important than currently believed. Not only did a substantial number of intramammary M. bovis infections cure, also new infections were detected at calving (Timonen et al., 2017). This might signify transmission in the dry period or reshedding of subclinically infected animals around calving. Having a calving pen was a protective factor for a positive M. bovis herd status, and likely more intense contact around calving increases within-herd shedding (Gille et al., 2018). Because successful treatment of clinical and subclinical M. bovis mastitis is questionable, this is not practiced.

Culling policy
The main difficulty to develop an efficient control program lies in the culling policy. Removal of severe clinical cases is an easy decision given the poor prognosis for production, but becomes a lot harder for mild and subclinical cases. There is only one observational study comparing herds culling and not culling after an M. bovis outbreak available. Most herds cleared M. bovis in the cows within 1 month and culling was not associated with hastened clearance (Punyapornwthaya et al., 2012). Unintentional culling of cows with mycoplasma mastitis in the control herds could not be excluded in that study. Culling decisions can be taken based on PCR, culture or antibody ELISA results. Given its contagious capacity, the number of animals exposed to M. bovis after introduction in a herd is very high. As a consequence, a substantial number, if not all, will develop antibodies. However, this does not necessarily mean that these animals are chronically infected or carriers. Despite that the actual prevalence of carrier animals has not been determined, available follow-ups of natural outbreaks show that this number is limited. Therefore, making culling decisions based on the presence of antibodies, independent from which ELISA is used, cannot be recommended. It will result in over culling with substantial economic consequences. Given the irreversibility of the decision, the most sensitive and specific test, namely PCR, is preferred for culling decisions. However, there are some drawbacks. First, a substantial number of cows appears to be able to clear the infection given the high number of seropositives compared to PCR positives. Therefore, PCR testing and immediate culling in the acute phase of the outbreak could also result in over culling. Based on observations from natural outbreaks, PCR based culling starting two months after the outbreak will likely avoid over culling. Also, this recommendation is questionable, since an Estonian study showed that 86.7% of M. bovis positive cows at dry off cured during the dry period (Timonen et al., 2017). Second, hygiene during sample is very important as carryover of bacterial DNA via the milking unit, milk
meter or sampling technique can occur, resulting in false positive PCR results (Mahmmod et al., 2017). Regardless the test, milk is the recommended medium to be tested, since vaginal samples were only positive in 18.8% of the animals diagnosed with *M. bovis* mastitis, and nasal and eye samples in none (Hazelton et al., 2018b).

**Control in calves and youngstock**

In 88.2% of 17 Finnish outbreaks starting with a mastitis index case, *M. bovis* could be isolated from nasopharyngeal swabs in the calves, and, as mentioned above, the infection persisted for over 1.5 years in 31.6% of the herds (Vahanikkila et al., 2019). In the authors’ experience, crucial elements for an effective control in calves are (1) termination of raw milk feeding, (2) individual housing until 8 weeks old (legal limitation in the EU (EC 2008/119/EC)), (3) metaphylactic antimicrobial therapy early in the disease course and (4) eradication of bovine viral diarrhea virus (BVDv), if present. Milk from infected cows is a primary source of infection for calves and the oral infection route is most efficient (Maunsell et al., 2012). Milk replacer or pasteurization are options. Especially young calves are more susceptible to develop severe disease; hence, the infection is postponed by prolonged individual housing. Co-infection of *M. bovis* and BVDv resulted in more chronic, unresponsive disease in veal calves and feedlots (Pardon et al., 2012). Given the contagious nature, metaphylactic therapy might be rational for *M. bovis* in an outbreak situation. Timing is crucial, and results are better when initiated early. In a natural outbreak of *M. bovis* pneumonia in beef young stock, florfenicol and oxytetracycline resulted in cure rates of 100% and 98.4%, as evidenced by thoracic ultrasonography (De Cremer et al., 2019). Using this ultrasound-based follow up, the required treatment length could be reduced from 7 days to 3.6 and 4.6 days for florfenicol and oxytetracycline, respectively (De Cremer et al., 2019). Trends in antimicrobial resistance in *M. bovis* are relatively stable over the years, and with the exception of the macrolide family, minimal inhibitory concentrations (MICs) are generally low for frequently used molecules like florfenicol, tetracyclines as well as for the critically important fluoroquinolones (Klein et al., 2019). Problematic is the absence of CLSI breakpoints, which hampers every clinical interpretation of MICs for *M. bovis*. In the authors’ experience, the above-mentioned approach for control in calves and young stock is clinically effective, limiting economic losses and assuring animal welfare. Whether the infection is cleared from the farm by this approach or only timely suppressed is currently unknown.

**Internal biosecurity**

Only a limited number of within-farm factors have been associated with a positive *M. bovis* herd status in dairy cattle, namely use of a breeding bull and not using a calving pen (Gille et al., 2018). Testing both teaser and breeding bulls might be beneficial, especially in herds with persisting infections. PCR testing of semen on samples from multiple days is likely the most sensitive approach. In beef cattle shared pen water was a risk factor for sero-increase, but this factor is difficult to adjust in dairy cattle, since shared water is standard (Schibrowski et al., 2018b). Given that calves are infected in many more herds than those facing clinical *M. bovis* mastitis, special attention should be given to avoid transfer of the pathogen from the calves to the dairy cows. Separate calf rearing units with own clothing and boots, combined with rinsing, disinfection and respecting walking lines are possible measures. However, herd immunity plays a role since many herds are actually antibody, or even antigen positive in the dairy cows, without noticing any clinical abnormality in this group. Potentially, severe outbreaks of *M. bovis* mastitis require specific strains or simultaneous exposure to immunosuppressing factors before an outbreak of clinical mastitis occurs.
**Prevention**

Prevention signifies all measures taken to avoid a pathogen to enter a currently negative herd. It is beyond the scope of the present paper to review current advances in vaccination against *M. bovis*. The overall conclusion is that an effective, commercially available vaccine is still absent (Perez-Casal et al., 2017), and therefore prevention is currently limited to biosecurity measures. Fortunately, airborne spread appears to be insignificant, making biosecurity measures useful. The two dominant risk factors for *M. bovis* are purchase and large herd size (Fox et al., 2003, Maunsell et al., 2011). Even in middle-sized (61 animals), family owned dairy’s in Finland, persistence of the bacteria was linked with increasing herd size (Vahanikkila et al., 2019). Larger herds often need to purchase to enlarge, but also in these herds a larger turnover in susceptible calves being born exists, facilitating persistence of the infection in the herd. Maternal antibodies can be detected until 3 months, but it is unknown whether they are protective, especially since most clinical infections, also in endemically affected herds, occur between 2 and 8 weeks of age (Maunsell et al., 2011).

Purchase control is the crucial measure to prevent *M. bovis* introduction in a herd. As with culling, intermittent shedding seriously hampers adequate decision making. Since the main interest is in having no false negative results, tests with the highest sensitivity are preferred. Also, parallel testing, as in using two tests at the same time and considering animals positive if one of them is positive, will increase sensitivity of the diagnostic procedure. Available information includes both testing of the animal itself as information on the infection status of the herd of origin. Determining the infection status of a herd can be done using different methods, with different reliability (see under monitoring). Different testing scenario’s (different tests, different combinations of tests and different starting prevalence) can be visually evaluated using Fagan Nomograms. In the absence of a well-designed study determining sensitivity and specificity of real-time PCR on milk, we used information from a recent Bayesian latent class evaluation of PCR on broncho-alveolar lavage fluid (sensitivity= 94.8% and specificity= 88.9%) (Bokma et al., 2019). In a scenario with a pretest probability of 5%, a positive PCR test stands for a probability of 40% that the animal is *M. bovis* positive. In the case of a negative test result, there is less than 0.5% probability that the animal is positive. Using culture, the probability of being *M. bovis* positive would be 40% and 2% in case of a positive and negative test result, respectively. Using the BIO-K302, in case of a positive result we are 20% certain that the animal has *M. bovis*, whereas in case of a negative result, there is still a probability of 3.5% that the animal is *M. bovis* positive. Using PCR and the BIO-K302 ELISA in parallel results in a sensitivity of 97.4% and specificity of 79.7%. Animals testing positive have a probability of 20% of being *M. bovis* positive, whereas animals testing negative have less than 0.2% probability of harboring *M. bovis*. In dairy cattle, milk is the preferred medium given the low prevalence of *M. bovis* on nasal, eye and vaginal mucosae in these animals (Hazelton et al., 2018b). Purchase of a breeding bull might signify a larger risk, and PCR testing of sperm should be considered (Gille et al., 2018, Haapala et al., 2018). In a recent study, only 3.8% of recently infected bulls were *M. bovis* positive in semen, of which none showed clinical signs (Hazelton et al., 2018a). Special attention should also be given to purchased animals with respiratory signs. Using thoracic ultrasonography might improve detection rates, as a substantial number of animals suffers from subclinical pneumonia (van Leenen et al., 2019, De Cremer et al., 2019). Regardless of the sample tested, the issue of intermittent shedding hampers every testing procedure. Unfortunately, there is hardly any information on shedding patterns in animals chronically infected by *M. bovis* available. Repeating the tests on samples from different days or exploration of other sampling sites (e.g. tonsils) might further improve diagnostic accuracy. Quarantine of purchased
animals, while awaiting tests, is recommended, also to avoid infection after samples have been taken.

Next to purchase control, other external biosecurity factors can be taken to reduce the risk of intromission through fomites and persons. This can be largely blocked by the use of herd-specific clothing and materials, combined with on farm cleaning and disinfection. Purchased colostrum can be heat-treated (60°C; 60 min.) (Donahue et al., 2012).

Monitoring and Surveillance
There is great interest in determining the herd status for *M. bovis*, both for national surveillance as for purchase purposes. Preference is given to easy, accessible samples, hence the popularity of the use of PCR and antibody ELISA on BTM. Recently, in a latent class model the sensitivity and specificity of the BIO-K302 ELISA for BTM were 60.4% and 97.3%, respectively (Nielsen et al., 2015). However, in a recent prevalence study using this ELISA none of the PCR positive herds was antibody positive, demonstrating the added value of combining both tests (Gille et al., 2018). Despite that this approach might represent to some extend the situation in the lactating animals, it is unlikely to be representative for the herd status. In natural outbreaks, BTM returned PCR negative within a month, whereas the infection could be demonstrated in nasopharyngeal swabs from the calves in the majority of the herds for more than a year (Vahanikkila et al., 2019). Using antibody ELISA has the advantage of a longer timeframe to assess the animals, and to follow the evolution of the herd. Usefulness highly depends from the ELISA used. Using the Bio-K302 ELISA, antibodies remained elevated in BTM for 8 months after the index case, whereas this was not reported for the MiLA ELISA (Parker et al., 2017a, Vahanikkila et al., 2019). BTM is one option, but given that mainly cows with mastitis are antibody ELISA positive in milk (Petersen et al., 2018a) antibody ELISA on serum might be preferred. On serum samples huge differences exist in the results of the BIO-X ELISAs and the MiLA ELISA, with the first having far less seropositives (Vahanikkila et al., 2019). With the MiLA ELISA antibodies in serum persisted for two years in adults, also in herds clearing the infection, questioning the benefit of monitoring these animals (Vahanikkila et al., 2019). In calves the situation is better, and serology with the MiLA ELISA followed infection patterns, whereas the Bio-X ELISA was not recommended in calves younger than 3 months (Vahanikkila et al., 2019).

Also, when restricted to calves, antibody ELISA remains an indirect measure of following infection. Based on current knowledge, combining PCR and antibody ELISA on BTM with PCR/culture of nasopharyngeal swabs of calves is likely the most accurate method to determine the herd status for *M. bovis*. In addition, PCR/culture should be done in cases of clinical mastitis and pneumonia to assure early detection, allowing maximum benefit from biosecurity measures. Underestimation of pneumonia in dairy calves and hence of *M. bovis* remains one of the key issues and sensibilization of farmers is needed to better control the pathogen (Gille et al., 2018, van Leenen et al., 2019). Whether herds truly clear Mycoplasma or whether a balance between herd immunity and the pathogen is retrieved is currently unknown, but crucial knowledge for every national surveillance or eradication program.

Conclusions
Substantial advances in *M. bovis* diagnostics have been made in recent years opening different routes for better control and prevention. Essential remaining research gaps are shedding patterns in carrier animals and on farm persistence in animals or the environment. It is clear that the solution of an *M. bovis* problem herd lies beyond application of the contagious mastitis related measures from the 10-point plan. Essential elements to add are control in
calves and young stock (no cow’s milk; prolonged individual housing; antimicrobial metaphylaxis and BVDv free status) and separate housing from dairy cattle combined with customized internal and external biosecurity. To accurately determine herd infection status, monitoring programs will have to include samples from calves.

References


