What are the keys to the prevention and control of Mycoplasma in dairy herds?

Disease caused by *Mycoplasma* species in Australian dairy herds has been diagnosed across all major dairying regions within Australia. The herd-level prevalence of *Mycoplasma bovis* has been estimated at between 0.1 and 3.5% in south-eastern Australian dairy herds (Penry et al., 2014). The consequences of an outbreak on a farm can be extremely costly so it is imperative that those working within the industry remain up-to-date with current recommendations for the prevention, diagnosis and control of disease caused by *Mycoplasma* species.

Mycoplasma species belong to a class of bacteria known as Mollicutes (Latin for "soft skin") which are unique because of their absence of a cell wall. This characteristic explains why many *Mycoplasma* species capable of causing mastitis do not respond to commonly used antimicrobials that rely on a mode of action involving interference with cell wall function and synthesis (Nicholas, 2004).

M.bovis **is the most common pathogenic species but others have been associated with disease in Australia. The major PCR test available does not detect these other species.**

Mycoplasma Species

The most common *Mycoplasma* species associated with disease in cattle is *Mycoplasma* bovis, however other species are also reported to cause disease in cattle including *Mycoplasma alkalescens, Mycoplasma arginini, Mycoplasma bovigenitalium, Mycoplasma bovirhinis, Mycoplasma californicum, Mycoplasma canadense, Mycoplasma dispar, Mycoplasma Leachii* sp. nov. (previously known as *Mycoplasma serogroup 7*) and *Mycoplasma F-38* (Fox et al., 2005; González and Wilson, 2003; Manso–Silván et al., 2009). In addition to *Mycoplasma bovis, Mycoplasma californicum, Mycoplasma alkalescens and Mycoplasma Leachii* sp. nov. (previously known as Mycoplasma serogroup 7) have been isolated from diseased cattle in Australia. It is important for advisors to consider these other *Mycoplasma* species because diagnostic tests currently available in Australia are mostly limited to detecting *M. bovis* or do not initially determine the individual species isolated in culture. There are also non-pathogenic Mollicutes (e.g. *Acholeplasma species*) that may be identified through culture. The commonly available PCR test does not detect species other than *M. bovis*.

Genetic characterisation of *M. bovis* in Australia shows very little variation in isolates collected from various geographical locations over a 9 year period from different anatomical site of clinically and subclinically infected animals (Parker et al., 2016b). This suggests host and environmental factors play a significant role in determining the host pathogen outcomes.

Disease Manifestations

In Australia, while mastitis is probably the most common disease associated with mycoplasma infections, other manifestations may be present. These include arthritis/tenosynovitis, pneumonia, keratoconjunctivitis, otitis, meningitis, endometritis, salpingitis, oophoritis, seminovesiculitis, infertility and abortion (McAuliffe et al., 2004; Nicholas and Ayling, 2003; Pfützner and Sachse, 1996). In adults, arthritis/tenosynovitis and pneumonia are the most common presentations after mastitis while polyarthritis/tenosynovitis, otitis, keratoconjunctivitis and pneumonia are common in calves.

Transmission

Mycoplasma species are considered highly contagious pathogens (González and Wilson, 2003). They colonise mucosal surfaces such as the mammary gland, nose, respiratory tract, eye, ears, vagina and prepuce (Fox et al., 2005). Disease is commonly transmitted via secretions from these surfaces. The main route of infection is intramammary, via the teat canal. Transmission to other quarters is most common via contamination from the initial infection although hematogenous spread has been suggested (Biddle et al., 2005). Aerosolisation of nasal secretions is also another common route of transmission. Contaminated clothing and equipment may serve as fomites infecting susceptible stock (González and Wilson, 2003). Ingestion of contaminated milk is a major source of infection for calves.

Diagnosis

In the initial stages of a herd infection, changes to mastitis rates may go unnoticed. Often the first indication that infection is present is an unusual number of calves with polyarthritis that is more severe and non-responsive than usual.

To perform the appropriate diagnostic tests, a veterinarian, advisor or producer first needs to be aware of the common clinical signs that suggest disease may be caused by a *Mycoplasma* species. In the milking herd, these include:

- Poor response to routine mastitis treatment
- Clinical mastitis in multiple quarters of the same cow
- Milk from infected glands often has a watery 'urine-like' appearance with flaky sediment (although abnormal milk appearance can vary)
- Mastitic cows presenting with enlarged udders that yield very little milk
- Sudden occurrence of swollen limbs and/or joints
- Rapidly increasing number of mastitis cases in the hospital herd

On farms that feed 'hospital herd' milk to calves, disease should be suspected if calves display clinical signs such as droopy ears, head tilt, swollen limb joint/s and/or difficulty breathing.

Poor response to routine treatment of clinical mastitis and presence of increased numbers of multiple quarter cases should be triggers for further investigation.

The following factors regarding *Mycoplasma* species provide challenges for the detection of infected animals. Cows with clinical intramammary infections often shed >106 colony forming units (CFU) per ml milk. On the other hand, the number of organisms shed and the frequency of shedding is extremely variable with subclinical infections. One study assessing the daily shedding patterns of 10 infected cows over a 28 day period using culture, found no mycoplasma organisms were shed 29% of the time, 102-104 CFU/ml were shed 10% of the time, 104-105 CFU/ml were shed 1% of the time and >105 CFU/ml were shed 60% of the time (Biddle et al., 2003). Another study monitoring cows with chronic *M. bovis* intramammary infections for an extended period of time, included a cow that did not shed M. bovis for 56 days (González and Wilson, 2003). The end concentration of mycoplasma organisms within a bulk tank milk sample will be affected by dilution and this could therefore limit detection. The shedding pattern for different *Mycoplasma* species could vary.

Known infected milk samples, have been diluted up to 1,000 fold and still returned positive PCR results. This suggests that if a cow is shedding and contributing milk to the vat, the PCR test will return a positive result even with very few infected cows in a large herd.

Diagnostic tests

Culture – specialised media is required so it is important for the sample submitter to specifically request a mycoplasma culture. *Mycoplasma* species are slow growing, so a result generally takes a minimum of 7 days for assessment, sometimes longer. A delay in initial diagnosis can be costly as new animals can become infected while waiting for a diagnosis, so PCR is often preferred even though it is more expensive.

Taking a representative sample is important along with appropriate sample handling, storage and transport. Minimise contamination by preparing the sample site using alcohol wipes. In cases of mastitis, milk from the infected quarter should be collected into a sterile sample tube. A fine needle aspirate should be collected from swollen joints into a plain tube. Swabs can be taken from affected mucosal surfaces or during a post mortem and transported in AMIES media. Samples should be refrigerated following collection and express shipped fresh on ice to the laboratory. Samples stored for any longer than 2 days should be frozen (Maunsell et al., 2011). Be mindful of sending samples close to a weekend which could potentially result in the samples being compromised if transport is delayed.

It is important to determine the species of any growth on specialised mycoplasma agar. If mycoplasma culture reports growth but the clinical signs are not consistent with a mycoplasma infection, seek speciation as it could be an *Acholeplasma* sp. *Acholeplasma laidlawii* is an important non-pathogenic contaminant (Britten, 2006) that is closely related to *Mycoplasma* species, however, indistinguishable on gross morphology. This phenomenon appears more common when collecting samples during wet muddy conditions.

PCR – At present, the only *Mycoplasma* species for which there is a commercially available test is *M. bovis*. This test measures the quantity of M. bovis DNA within the sample. The sample can be conducted on milk from an individual cow or a sample from the bulk milk tank. Herd level specificity of the Pathoproof™ PCR for mycoplasma is likely to be >97%, however, the sensitivity for *M. bovis* has not been determined precisely but is unlikely to be 100% (Penry et al., 2014). PCR results with a high Cycle Threshold (CT) value should be interpreted in combination with clinical signs. Retesting may be indicated in the absence of clinical signs. This test will only detect *M. bovis*. If clinical signs are consistent with a mycoplasma infection but the *M. bovis* PCR is negative, additional diagnostics must be sought as it could be another species like *M. californicum, M. alkalescens, or M. leachii*. A species specific multiplex probe PCR for M. bovis, *M. bovigenitalium and M. californicum* exists, however, it is not yet commercially available in Australia (Parker et al., 2017). A *Mycoplasma bovis* PCR costs approximately 3 to 4 times that of culture. Samples from individual cows can be pooled to save costs. The actual cost saving will be determined by the prevalence of positive samples and pool size. Preservative can be used in milk samples submitted for PCR (Pinnow et al., 2001) if this is part of the laboratory sampling protocol.

While freezing reduces the number of viable organisms in a milk sample, most clinical cases excrete sufficient numbers for this to have only a small effect on the rate of positive cultures.

Serology - Enzyme-linked immunosorbent assay (ELISA) kits are commercially available (Bio X Diagnostics) to assess *M. bovis* antibodies in milk and serum, however, detection of seroconversion is poorly associated with infection status or disease of an individual animal – it simply measures exposure (Fox et al., 2005). It is possible that ELISA could be used to measure and monitor herd prevalence, however, preliminary research suggests it is not an appropriate test for evaluating individual animals. The use of a bulk tank milk (BTM) sample to detect herds with past exposure to *M. bovis* has proven useful for surveillance and biosecurity reasons, and is more likely to return a positive result than BTM culture or PCR. This is because the ELISA is detecting exposure to *M. bovis*, not shedding. It therefore may have a role as a complimentary tool with culture and PCR.

Research Priority - High

Determine sensitivity of commercially available PCRs and detection limits in a BTM sample.

Testing the "carrier animal" – There is currently no test that will reliably detect an animal that may be carrying *M. bovis* that is exhibiting no clinical signs and is not shedding the organism in milk. From 16 cows with recent *M. bovis* mastitis detected via PCR, *M. bovis* was unable to be recovered using nose or eye swabs and it was only isolated from the vagina of 18% (3/16) (Hazelton et al, 2017). Sampling these sites in unlikely to reliably detect subclinical infection in non-milking stock.

Research Priority - High

Determine ELISA sensitivity and specificity.

As a general principle, the use of more than one test is likely to increase both the sensitivity and specificity of a biosecurity assessment.

Accurate assessment of non-milking stock for subclinical infection.

Control

There appears to be three phases following the introduction of mycoplasma into a naïve dairy herd that reflect the dynamics of pathogen exposure, host immunity, and management knowledge and experience. Environmental stressors such as poor weather, nutrition, management practices, laneway conditions and housing facilities commonly precede outbreaks and particular disease presentations. For example, slippery concrete and poor freestall comfort leading to excessive time standing in an intensive facility has been observed to contribute to the tenosynovitis/arthritis form of clinical disease. It is possible that wet and muddy conditions can increase the number of environmental mastitis cases, in turn increasing the size of the hospital herd and pressure on staff, leading to routine procedural failure and rapid transmission of a concurrent Mycoplasma outbreak.

Phase 1 – Initial outbreak: This is typically the most costly phase reflecting the spread of a contagious pathogen in a naïve herd in the face of a management team that is not familiar with the disease and its transmission. During this phase of the disease the focus is on identifying and isolating/culling clinically infected cows and blocking the transmission of disease to young stock.

The cost of this phase is largely influenced by the time taken to establish a diagnosis and initiate control interventions. It is important that diagnostic test results are viewed in light of the clinical presentation of animals. False negative results are possible if the organism has died during transport to the laboratory for culture or if the *Mycoplasma* species is not *M. bovis* when using the PCR.

Cow to cow spread commonly occurs at milking time, particularly in the "hospital" herd. A review of the hygiene of milking practices is necessary (Fox et al., 2005). The 'hospital herd' should be the initial focus as this is where the majority of infected cows are usually located and subsequently the rate of transmission to naïve cows is greatest. The following control measures should be implemented immediately upon diagnosis or suspicion of an outbreak:

Initial efforts to determine the prevalence of M. bovis in the herd should be targeted at the cows most likely to be infected. This includes cows in the hospital herd and those with clinical signs of mastitis.

- Review of the herd's milking management practices as soon as possible. Ensure that transmission of infection from cow to cow in the milking shed is minimised. Refer to Technote 5: Use good milking technique and a consistent routine and Technote 8: Practice good hygiene during milking.
- Stripping cows to identify clinical cases should be performed judiciously as there is a high potential for aerosol contamination and spread during this procedure. If performed, milkers should rinse and disinfect gloves with disinfectant between every cow.
- A complete review of the herds milking machine function should be carried out. Milking machine factors that favour the transmission of cow associated pathogens should be controlled. Refer to Technote 6: Monitor and maintain milking machine function.
- Initially it is advisable to sample and test every quarter of every cow within the 'hospital herd' (a composite milk sample is adequate).
- All new clinical cases should have individual affected quarters sampled for testing prior to treatment.
- Animals determined to be infected should be segregated and culled after relevant drug withhold periods.
- Time is of the essence when identifying infected animals, so PCR is considered the preferred diagnostic test for herds infected with *M. bovis*, despite the extra cost. It is advisable to communicate regularly with your laboratory to minimise delays. The discussion should include apprepriate sampling techniques, the type of samples being sent, the number of samples expected, sample handling methods, the option of pooling samples and pool sizes.
- Cows with clinical mastitis that are determined not to be infected with mycoplasma and do not respond to treatment should be resampled prior to initiating further treatment.
- DO NOT RUN RECENTLY CALVED COWS WITH COWS IN THE HOSPITAL HERD (Britten, 2006; Fox, 2012). The hospital cows represent the highest risk group and recently calved cows the most susceptible group.
- If a milking cluster is to be used more than once on cows within the 'hospital herd' it is recommended to immerse the clusters in a bucket of disinfectant (eg.1% iodophor) and backflush the contents (González and Wilson, 2003).
- To minimise the risk of transmission, particular care must be taken when administering intra-mammary antibiotics. Systemic antimicrobials can be considered for mastitis treatment at the peak of an outbreak. Outbreaks have been associated with contamination introduced during the administration of dry cow therapy (Mackie and Ball, 1986).
- Feeding mycoplasma contaminated colostrum and/or milk to calves is an effective method of transmitting disease. DO NOT POOL COLOSTRUM – pooling colostrum increases the number of calves exposed by an infected cow by distributing contaminated colostrum to more calves. DO NOT FEED WASTE MILK

TO CALVES. Calves ingesting mycoplasma contaminated milk are likely to develop disease so prompt decision making is important. Some herds choose to feed milk replacer or install a pasteuriser (Butler et al., 2000). Preliminary research in the laboratory demonstrates milk acidification may be a useful tool in the future (Parker et al., 2016a). Handling safety of acidifying products needs to be carefully assessed.

› Colostrum may be heat treated at 60 degrees for 60 minutes to reduce the risk of transmission without significantly effecting the colostrum quality (Godden et al., 2006).

Phase 2 – Post outbreak: During the initial outbreak, serological studies suggest that clinical disease is likely to reflect the tip of the iceberg with a large number of cows exposed to mycoplasma without developing disease. This may in part reflect the route of infection. Sub-clinically infected cows may be colonised at any mucosal surface. It is also possible that cows with sub-clinical mycoplasma mastitis remain in the milking herd.

Herd level shedding of mycoplasma can be monitored by screening the bulk tank and hospital herd milk using PCR. The frequency of follow up testing will be determined by the progress of control. Ongoing testing also serves as a reminder to farm managers and staff that this disease requires continued vigilance. Once all infected cows have been removed, ongoing weekly monitoring of the bulk tank is recommended (González and Wilson, 2003). After a series of negative weekly results are obtained, extending to fortnightly or monthly testing to reduce costs can be considered. Identification of a positive bulk tank indicates an infected cow (clinical or sub-clinical) contributed to the tank. Individual sampling of all milking cows is expensive and is often unrewarding. Closer examination of cows that have recently returned from the hospital herd and high cell count cows is likely to be more rewarding. If milking hygiene is good, disease transmission in the milking herd may be minimal.

Immediately after the post outbreak phase, it is not uncommon to have intermittent cases and occasional smaller clusters of clinical cases. Disease clusters generally reflect breakdowns in detection of clinical disease and milking hygiene practices. Ongoing surveillance of all clinical cases of mastitis is warranted during this phase. From a cost management perspective, this can be achieved by pooling individual cow samples or monitoring the hospital herd tank milk. It is common for staff to become adept at detecting and distinguishing most clinical cases, however diagnostic support is required to avoid over or under diagnosis.

Phase 3 – Endemic infection: Currently there is no method for eradication of Mycoplasma from a herd. As cows may be sub-clinically infected and carry the organism at different mucosal sites, attempts to eradicate the organism through culturing milk samples from all cows is not effective. Herds with a history of mycoplasma disease usually reach a point where disease is rare if good husbandry practices are in place. Despite this, disease outbreaks may be observed intermittently. When these occur, they tend to be triggered by stressful events such as adverse weather conditions, nutritional, or physical stress (Bushnell, 1984; Jasper, 1979). These outbreaks are typically observed around the time of calving. Often first calf heifers are over represented possibly reflecting their naïve status. Addressing the underlying stressors contributing to compromised immunity is an important aspect of managing these outbreaks. Management memory helps to mitigate the magnitude of these outbreaks when staff are familiar with the disease. Loss of management memory contributes to disease risk when there is high staff turnover.

Prevention

Possible sources of infection for a herd include:

- Introduced livestock (cows, calves and bulls)
- Equipment (particularly equipment that contacts the mucosal surfaces of stock e.g. AI guns)
- People (service providers, AI technicians, veterinarians)
- Biologics (semen, embryos)

The most common source of infection reported is introduced livestock. Maintaining a closed herd is the best way to prevent entry of *Mycoplasma* species onto a disease naïve farm. The risk posed by semen and embryos is generally considered low. Service providers and their equipment reflect variable risk depending on their contact with infected farms and their adoption of sound biosecurity practices. It is reasonable and wise for farms to discuss their biosecurity expectations with service providers to manage this risk.

Mitigating risk associated with purchasing stock

There are a number of strategies a farm can incorporate into their biosecurity protocol to minimise the risk of introducing mycoplasma infection.

- Clarify disease history of source farm, particularly with respect to the common presentations of *M. bovis*. This will include clinical mastitis rates, BMCC history and calf disease, especially joint ill. Determine if there has been any previous surveillance for *M. bovis*.
- Request PCR testing of bulk and hospital milk samples. Infected cows are more likely to be in the hospital herd so waste milk is likely to be a more sensitive method of identifying infected herds than bulk tank milk. While the PCR is likely to be more sensitive than culture, the following principals still apply when attempting to determine if a herd is infected. Due to intermittent shedding patterns of infected cows, the probability of identifying an infected herd by culture, on a single BTM sample is 33-50% (Wilson, 1999; González and Wilson, 2002). If a minimum of 3 BTM samples are collected, 3 to 4 days apart and all are negative on culture for *Mycoplasma* species, the probability that cows contributing milk to the tank are negative for *Mycoplasma* species is 70% (González and Wilson, 2002). Collecting 5 BTM samples 3 to 4 days apart increases the sensitivity to 97%. The rationale behind this sampling protocol is due to intermittent shedding patterns of infected cows which often fluctuates in 1-week cycles (Wilson et al., 2009). In a study that included 19 herds with a history of *M. bovis* infection, BTM PCR was rarely positive (Parkeret al, 2017).
- Evaluating the antibody level in BTM using ELISA can be used to measure past exposure to *M. bovis* and is more likely to return a positive result than BTM culture or PCR. From a biosecurity perspective, a screening test is a preliminiary risk assessment, not a definitive test of infection status. BTM ELISA used as a screening test will give a better indication of *M.bovis* exposure in a herd compared to culture and PCR. Following an initial *M. bovis* outbreak, the BTM ELISA value is highest in the first 8 months before significantly decreasing. An association also exists with calving, with BTM ELISA values highest 5-8 weeks following the commencement of the calving period in split- and seasonal-calving herds (Parker et al., 2017b).
- When purchasing cows in milk, assessing a single somatic cell count is not considered a useful tool for screening potential herd additions for contagious mastitis pathogens including *M. bovis* (Biddle et al., 2003). When using culture, sampling individual quarter milk samples is the most sensitive (24%) method for detecting an infection (González and Wilson, 2002). However, this may not be considered practical or affordable when purchasing large numbers. Therefore, pooling composite milk samples from a group of animals is a useful first step when screening for *Mycoplasma* species. This technique has limitations whereby positive animals may be missed due to intermittent shedding or dilution of a sample below the detectable threshold of culture (Biddle et al., 2003). If a milk sample is unable to be obtained as is the case when purchasing dry cows, heifers

Conducting a PCR test on a herd's bulk milk is a useful screening test for *M.bovis*. Intermittent shedding and the fact that not all cows in the herd will be contributing to the vat on any given day, means that a negative result is NOT a guarantee that the herd is free of the organism.

Research Priority - Moderate

Studies on the probability of identifying infected herds using PCR testing of BTM samples.

or calves, it is recommended to quarantine these animals until they calve, after which a sample can be collected and tested prior to mixing with the new herd. As sub-clinically infected animals can be shedding low numbers of organism (Biddle et al., 2003), it is recommended to send fresh samples to the laboratory for culture to increase the chance of detection. PCR is an alternative to culture, however, will only detect *M. bovis*.

› Care should be taken when considering the purchase of bulls from a herd with a history of mycoplasma infection. *Mycoplasma* species have been isolated from the semen of bulls (Ungureanu et al., 1986). Culture and PCR methods for detecting infected bulls are limited by variable shedding. The risk can be minimized by purchasing bulls from herds with no history of Mycoplasma associated disease.

Treatment

Mycoplasma species recovered overseas have demonstrated *in vitro* susceptibility to a number of antibiotics such as tylosin, tulathromycin, tilmicosin, spectinomycin, lincomycin and oxytetracycline. However, in practice, no antibiotic treatment therapy is regarded to be effective for the treatment of mastitis (Boughton, 1979; Bushnell, 1984; Fox, 2012). Tulathromycin has been utilised to treat calves with pneumonia and arthritis. There are no effective therapeutics registered for the treatment of lactating cows.

Vaccination

Currently there is no registered vaccine against mycoplasma available for cattle in Australia.

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