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A review of mycoplasma diagnostics in cattle

Alysia M. Parker ⬤ | **Paul A. Sheehy** | **Mark S. Hazelton** | **Katrina L. Bosward** | **John K. House**

Sydney School of Veterinary Science, The University of Sydney, Camden, New South Wales, Australia

Correspondence
Alysia M. Parker, Faculty of Science, Sydney School of Veterinary Science, The University of Sydney, 425 Werombi Road, Camden, New South Wales 2570, Australia.
Email: alysia.parker@sydney.edu.au

Mycoplasma species have a global distribution causing serious diseases in cattle worldwide including mastitis, arthritis, pneumonia, otitis media and reproductive disorders. *Mycoplasma* species are typically highly contagious, are capable of causing severe disease, and are difficult infections to resolve requiring rapid and accurate diagnosis to prevent and control disease outbreaks. This review discusses the development and use of different diagnostic methods to identify *Mycoplasma* species relevant to cattle, with a particular focus on *Mycoplasma bovis*. Traditionally, the identification and diagnosis of mycoplasma has been performed via microbial culture. More recently, the use of polymerase chain reaction to detect *Mycoplasma* species from various bovine samples has increased. Polymerase chain reaction has a higher efficiency, specificity, and sensitivity for laboratory diagnosis when compared with conventional culture-based methods. Several tools are now available for typing *Mycoplasma* spp. isolates, allowing for genetic characterization in disease outbreak investigations. Serological diagnosis through the use of indirect ELISA allows the detection of antimycoplasma antibodies in sera and milk, with their use demonstrated on individual animal samples as well as BTM samples. While each testing method has strengths and limitations, their combined use provides complementary information, which when interpreted in conjunction with clinical signs and herd history, facilitates pathogen detection, and characterization of the disease status of cattle populations.

KEYWORDS
culture, ELISA, mastitis, PCR

1 | INTRODUCTION

Organisms in the *Mycoplasma* genus belong to the class Mollicutes which are characterized by their lack of cell wall, low G + C content (23%–40%) and small genome size (0.58-1.4 Mbp).[1] Isolated from cattle in 1898 as the first *Mycoplasma* species, *Mycoplasma mycoides* subsp. *mycoides* (formerly known as *M. mycoides* subsp. *mycoides* small colony)

was characterized as the causative agent of contagious bovine pleuropneumonia.[2] After reaching near global spread in the 19th century, it was eradicated from most continents through "stamping-out" policies; however, it remains in many African countries.[3] *Mycoplasma bovis* is currently recognized as one of the most important and frequently isolated *Mycoplasma* species associated with disease in cattle worldwide.[4] *M. bovis* was first isolated in the United States of America in 1961 from a severe case of mastitis in a dairy herd experiencing an outbreak, affecting more than 30% of the animals.[5] Several other *Mycoplasma* species are of interest in cattle with varying degrees of clinical significance. These include *Mycoplasma (M) californicum*, *M. bovigenitalium*, *M. bovirhinis*, *M. bovoculi*, *M. leachii* (previously *Mycoplasma* sp. bovine group 7),[6] *M. dispar*, *M. canis*, *M. canadense*, *M. alkalescens*, *M. arginini*, and *M. wenyonii*.[7–10] Other closely related species belong to the

Acholeplasma genus (also members of the Mollicutes class) and are often isolated alongside *Mycoplasma* species. They are commonly considered contaminants[10] and of minimal clinical importance in cattle,[8] with no clear evidence of their ability to cause disease.[1]

Mycoplasma can cause several diseases in cattle including mastitis, arthritis, pneumonia, otitis media and reproductive disorders.[11] Clinical mycoplasma mastitis is often characterized by multiple affected quarters coupled with unresponsiveness to treatment.[12,13] Adults and calves can also be affected by arthritis and pneumonia, while otitis media is typically only observed in calves. All of these clinical manifestations may be observed concurrently with mycoplasma mastitis in the herd.[14,15] Mycoplasma has also been associated with reproductive disorders (vulvovaginitis, infertility, endometritis, dystocia) however these manifestations are less consistently reported.[16–18] The highly contagious nature of some *Mycoplasma* spp., their poor responsiveness to treatment and associated culling implications for affected stock make rapid and accurate diagnosis important for control and prevention of disease outbreaks. This review discusses the development and use of different diagnostic methods available to identify *Mycoplasma* species relevant to cattle (excluding *M. mycoides* subsp. *mycoides*) with a particular focus on *M. bovis*.

2 | MICROBIAL CULTURE

Traditionally, the identification and detection of *Mycoplasma* species affecting cattle has been performed via microbial culture. Because of their structural simplicity as organisms, mycoplasmas are unable to synthesize amino acids and have complete or partial inability to synthesize fatty acids. In order to meet this high nutritional demand, the media used to grow mycoplasmas is specific and often highly enriched with beef heart infusion, serum, yeast extract, peptone, and other supplements with buffering to a final pH of 7.3–7.8 required.[19] Isolation of mycoplasmas by culture can be compromised by the overgrowth of other faster growing bacteria, therefore, antimicrobials such as thallium acetate or antibiotics are incorporated into the media.[7] Inoculated culture media is incubated for 7–10 days at 37°C and 5% CO_2, resulting in the growth of micro-colonies with a morphological appearance of "fried eggs" for most *Mycoplasma* species, which are visible via light microscopy.[10] This appearance is because of the central portion of the colony embedding itself into the agar surrounded by a zone of surface growth.[19]

The limit of detection for culture has been reported as low as 272 cfu/mL in milk.[20] However, the common practice of inoculating plates with a 10 μL milk volume to get maximum use of the agar means that, theoretically, the minimum concentration of *M. bovis*, which can be detected by culture is 100 cfu/mL since it is unlikely to identify <1 colony in 10 μL.[21] When mycoplasma is present in low concentrations in milk, to concentrate the cfu/mL and improve recovery by culture, the milk can be centrifuged first and suspended in a small volume of saline before culturing.[22]

While identification of mycoplasmas via culture may be the traditional method because of its relative simplicity and low cost, it does have several limitations. Mycoplasmas cannot proliferate in milk,[23] therefore the organism can be easily overgrown by other types of bacteria present as contaminants or part of a mixed infection in the sample causing false negative results. Appropriate sample collection, handling, and storage are essential to prevent or minimize the overgrowth of other bacteria in the sample, and to maximize mycoplasma viability and growth on the diagnostic media. Samples should be collected aseptically into sterile tubes or containers[7] and then kept at 4°C, and transported to the laboratory for culturing as soon as possible. If culture cannot be performed within 2 days, samples should be frozen.[24] Freezing samples can cause a 1–2 $\log_{10}$ reduction on the recovery of colony-forming units of *Mycoplasma* spp. in milk samples.[25] The implications of this loss of recovery will be different for individual clinical samples (with a likely high concentration of organisms), compared to pooled or BTM samples (with a likely lower concentration of organisms). Ideally, frozen samples should be thawed at room temperature to ensure maximum recovery, with repeat freeze thawing causing a further decrease in viable organisms.[25] Mycoplasma recovery rate decreases with increased time before laboratory processing regardless of whether samples have been refrigerated or frozen, with best recovery rates achieved when fresh samples are inoculated to media and incubated within a few hours of collection.[25] If samples are not collected and stored appropriately, false negative culture results may be reported.

With appropriate sample collection and storage protocols, mycoplasmas still exhibits a slow rate of growth, with colonies not visible for a minimum of 5 days and sometimes requiring up to 10 days.[10] This is problematic because rapid diagnosis of infection is desirable to facilitate removal of infected animals from the herd to minimize transmission.[4] Therefore, pending diagnostic results, animals suspected of being infected should be segregated from both the main herd and the hospital herd containing nonmycoplasma related cases.

In order for mycoplasma infection to be diagnosed by culture, the animal must be shedding viable organisms at the time of sample collection. Intermittent shedding is frequently reported with chronic and subclinical mycoplasma mastitis cases, which can result in a diagnosis failure from a single sample. In a study sampling 10 chronically infected animals daily over a period of 28 days, *Mycoplasma* organisms were isolated from 71% of composite milk samples.[26] Gonzalez and Wilson[27] reported a period of 56 days without shedding of *M. bovis* in a cow with a chronic infection. In BTM samples, the estimated probability of identifying an infected herd from one BTM culture is 33%.[28] Therefore to increase the likelihood of mycoplasma detection in chronic or subclinical mastitis cases as well as in BTM samples, multiple samples should be taken over several days. Sampling recommendations for BTM samples suggest that at least 3 samples should be collected 3–4 days apart and cultured. If all samples are negative, the estimated probability of that herd being negative is 70%.[28] Group testing may also be performed such as string sampling in which milk is collected from the milking system from groups of cattle rather than the entire herd. This may allow the possible identification of infected subgroups or pens of animals. However, milk residues left in the pipelines from one group may confound the results of the following group and as such should be taken into account when interpreting results.[29]

While added antimicrobials generally inhibit the growth of other bacterial families, mycoplasma growth media has the potential to grow several organisms from the Mollicutes class. Some isolates grown from mycoplasma media are known primary pathogens while others are considered pathogens of lower virulence requiring greater host compromise to cause disease. For example, mycoplasma media also grows many *Acholeplasma* species, an environmental contaminant with little evidence of a pathogenic role in natural disease.[1] Given that most species of both *Acholeplasma* and *Mycoplasma* have a gross morphological "fried egg" appearance, differentiation by culture alone is not possible,[19] and can result in the reporting of false mycoplasma-positive samples. Digitonin sensitivity can be used as an additional step to distinguish *Mycoplasma* from *Acholeplasma* spp. On a paper disc saturated with 1.5% digitonin a large zone of inhibition will surround mycoplasma, with a small to nonexistent zone of inhibition for *Acholeplasma* species.[30] However, interpretation of digitonin sensitivity can be subjective. Ideally, species identification via polymerase chain reaction (PCR) should be performed on any culture positive isolates.

3 | MOLECULAR DIAGNOSIS

3.1 | Conventional PCR detection

The use of PCR to detect *Mycoplasma* species from various sample types has demonstrated a higher efficiency, specificity, and sensitivity for laboratory diagnosis when compared with conventional culture-based diagnostic methodologies.[31] Analysis via PCR involves the amplification of the DNA of the target organism,[32] and as such, the organism must be present in the sample with nondegraded DNA for amplification to occur. However, unlike culture, the organism does not need to be viable for detection, which should be taken into consideration if the target is viable organisms only. Assays have been developed to detect individual *Mycoplasma* species, while some have been developed to identify multiple *Mycoplasma* species, followed by post-PCR speciation. The development of conventional PCR methods to detect *M. bovis* began in the 1990s, targeting the 16S rRNA gene.[33,34] The 16S rRNA gene is one of the most common genes targeted for bacterial identification due its presence in all bacteria and its function remaining unchanged over time.[35] The 16S rRNA gene is a small subunit within prokaryotic ribosomes, containing highly conserved regions and variable regions, which can be species specific, making it useful for bacterial identification.[36] The limit of detection of these early PCR assays was reported as 4×10^2 cfu/mL in broth cultures,[33] and 5×10^2 cfu/mL in milk samples after a DNA extraction process.[34] Results were produced within 24 hours, rendering the PCR method more rapid than culture. However, while the specificity of these PCR assays targeting the 16S rRNA gene of *M. bovis* appeared to be adequate against most *Mycoplasma* species, less specific amplification was seen with *M. agalactiae*,[33] a species affecting small ruminants.[37]

As *M. bovis* is not the only *Mycoplasma* species of diagnostic interest in cattle, further development of PCR assays allowed the detection of other species. The 16S rRNA gene has been used as a target to develop species specific PCRs for *M. dispar*[38,39] and *M. bovirhinis*.[39] For the detection of multiple species, a PCR targeting the 16S-23S rRNA spacer region was designed to allow the identification of *Mycoplasma* spp. and *Acholeplasma* spp. contaminants in cell cultures.[40] The 16S-23S rRNA intergenic spacer region is a structural region situated between 2 ribosomal RNAs, which are essential for protein synthesis.[41] While this design was not specific for mycoplasmas associated with cattle, it provided an alternate and broader approach. After DNA amplification, the resulting product could then be digested and run on an agarose gel, with the banding pattern differentiating *Mycoplasma* spp. from *Acholeplasma* spp. A similar approach by McAuliffe, et al[42] targeting the 16S rRNA gene used *Mycoplasma*-specific primers followed by separation of the PCR products using denaturing gradient gel electrophoresis (DGGE). This approach enabled the identification and differentiation of 67 *Mycoplasma* species of veterinary and human significance, and was useful in detecting mixed cultures. A further study by Jozefova, et al[43] also used a 16S rDNA PCR/DGGE assay to identify 8 *Mycoplasma* species from cattle with clinical signs of respiratory disease. With the technique used in these studies however, the variation between some species and therefore the interpretation of speciation can be very subtle, making accurate and confident species specific diagnosis of mycoplasma difficult. This is an important process, as not all *Mycoplasma* spp. are considered pathogenic.

Another approach to identifying multiple *Mycoplasma* species was the use of several primer sets, each specific to the species of interest including *M. alkalescens*, *M. bovigenitalium*, *M. bovirhinis*, and *M. bovis*, with speciation determined by the product size on an agarose gel.[44] Using this method the limit of detection in milk was reported as 1.4×10^3, 1.7×10^2, 1.1×10^2, and 4×10^2 cfu/mL for *M. alkalescens*, *M. bovigenitalium*, *M. bovirhinis*, and *M. bovis*, respectively. This was lower than culture, which had a limit of detection of 1.4×10^4, 1.7×10^2, 1.1×10^3, and 1×10^3 cfu/mL, respectively. Specificity analysis was not reported. A more recent study validated a conventional multiplex PCR followed by sequence analysis to allow the identification and discrimination of several *Mycoplasma* and *Acholeplasma* spp. including *M. arginini*, *M. alkalescens*, *M. bovigenitalium*, *M. canadense*, *M. bovirhinis*, *M. californicum*, *A. laidlawii*, and *Acholeplasma oculi*.[45] This multiplex PCR included 3 assays targeting 4 separate genes. While the first assay (targeting *M. bovis uvrC* and *Mycoplasma/Acholeplasma* 16S rDNA) showed cross amplification with 10 non-*Mycoplasma/Acholeplasma* species from bovine sources, the second (targeting *Mycoplasma* 16-23S rDNA), and third assay (targeting *Acholeplasma* 16-23S rDNA) showed appropriate amplification and discrimination of isolates.

3.2 | Real-time PCR detection

While the PCR techniques discussed have proved effective, conventional PCR measures the amount of PCR product at the end of the cycles. Therefore, gel electrophoresis is required for results to be analyzed in the form of amplified DNA visualized as bands, requiring additional time and labor. As technology has improved, real time PCR was developed and soon utilized in mycoplasma detection. The main approaches for real time PCR utilized for diagnostics of *Mycoplasma*

species are SYBR green dye intercalation and fluorescent reporter probes.

The cyanine SYBR green dye binds to all double stranded DNA resulting in light emission measured at 520 nm when excited by light at a specific wavelength. As the PCR cycles progress, the quantity of target double stranded DNA increases and the amount of light being emitted from the dye increases proportionately, allowing detection of the PCR product in real time. As SYBR green is not specific to a target sequence, this provides a much cheaper real time PCR analysis option as specific oligonucleotide sequences do not need to be synthesized. However, because SYBR green does bind to all double stranded DNA, it can create an increase in background signal and reduced specificity compared with probe-based real time PCR methodologies.[46] This method has been less commonly adopted to detect mycoplasma in cattle; however, it was used to detect multiple *Mycoplasma* spp. in bulk tank milk samples, targeting the 16S-23S intergenic spacer region of the *Mycoplasma* genus.[47] In this study, speciation was performed after PCR amplification by high resolution meting analysis, with the amplified DNA of different species of mycoplasmas melting at slightly different temperatures because of different nucleotide sequences and lengths. When compared with traditional culture, this method was not shown to be significantly more sensitive; however, it did allow organism speciation with several samples containing more than one species. SYBR green technology has also been used to develop a species specific PCR to detect *M. bovoculi* in conjunctival swabs from dairy calves and beef suckler cows as part of a point prevalence study into infectious bovine keratoconjunctivitis.[48]

To achieve greater specificity, the fluorescent reporter probe method of real time PCR was developed, typically utilizing a hydrolysis probe. In addition to primer hybridization, the probe binds to a targeted region internal to the primer binding sites.[46] Because of hydrolysis probes being specific to the target sequence, this can greatly reduce the background signal and increase the specificity of the assay. Different probes can also be conjugated to different dyes and quencher molecules, allowing the possibility of multiplexing assays in a single reaction, saving time and reagents. However, the overall cost of the test will be more expensive from the use of multiple probes.[46] Because of its specificity, several novel real time PCR assays with probes for the detection of *M. bovis* have been developed.[20,21,49–51] Despite probe-based PCR allowing greater specificity, when targeting the 16s rRNA gene of *M. bovis*, cross-amplification of *M. agalactiae* can still occur.[21] Therefore, the use of alternative genes was investigated.

The *uvrC* gene has been demonstrated to be a better PCR target for *M. bovis*, with no cross-amplification with non-*M. bovis* species including *M. agalactiae*.[50,51] The *uvrC* gene encodes deoxyribodipyrimidine photolyase, an enzyme which is essential for replication as it is involved with DNA repair, making it a highly stable gene.[52] It is a well conserved but significantly different gene in both *M. bovis* and *M. agalactiae* making it a much more specific target gene than 16S rRNA,[53] however recent studies have since identified point mutations in the *M. bovis uvrC* gene.[54] Validation of the *uvrC* gene as a target for identifying *M. bovis* using real time PCR has been demonstrated on clinical samples from the lung, milk, joint fluid, nasal swabs, bronchoalveoolar lavage

fluid, tracheal wash fluid, and ear swabs of cattle.[50] In milk samples, lung samples and mycoplasma liquid media spiked with *M. bovis*, the detection limits were as low as 2.4×10^2, 2.4×10^2, and 2.4×10^1 cfu/mL, respectively.

The use of the *oppD/F* genes and an *M. bovis* specific probe has also been demonstrated to add a further element of specificity into a PCR assay.[20] The *oppD/F* genes encode an oligopeptide permease and are a member of the ABC-transporter family. It facilitates transport of short peptides across the bacterial cell membrane and is therefore essential for many processes.[55] The use of this gene for detecting *M. bovis* has been validated in milk samples and nasal swabs, with a limit of detection as low as 1×10^2 cfu/mL in milk.[20] A further study demonstrated the use of an *M. bovis* specific PCR targeting the *oppD* gene in conjunction with a DNA microarray to allow the identification of more than 70 *Mycoplasma* spp. in nasal swabs and trans-tracheal aspirations from veal calves.[56] With the combined use of diagnostic techniques, results allowed a greater understanding of the diversity of *Mycoplasma* spp. within the respiratory airways of veal calves.

The identification of other *Mycoplasma* species in cattle has also been further explored with probe-based real-time PCR assays including *M. californicum* and *M. bovigenitalium*, both of which are also consistently isolated from bovine samples.[57,58] A recent study developed 3 individual probe-based real-time PCR assays to detect *M. bovis*, *M. californicum*, and *M. bovigenitalium* in bovine milk and tissue samples.[49] Three different target genes were selected for each species. For *M. bovis* the targeted gene was *fusA* encoding for elongation factor G, which is required during the translation process of mRNA into proteins.[59] For *M. californicum* the target gene was *rpoB* encoding for RNA polymerase β subunit, which is an enzyme responsible for catalyzing the synthesis of RNA during transcription in which DNA is copied.[60] Lastly for *M. bovigenitalium*, the target gene was the 16S-23S rRNA intergenic spacer region. Specificity assessment demonstrated no cross-amplification with other Mollicutes. The detection limit in spiked milk was as low as $1 \times 10^1 \pm 0$ (SE) cfu/mL, $2.24 \times 10^1 \pm 2 \times 10^1$ (SE) cfu/mL, and $2 \times 10^1 \pm 0$ (SE) cfu/mL for *M. bovis*, *M. californicum*, and *M. bovigenitalium*, respectively. This limit of detection was significantly more sensitive than previously described PCR assays,[20,50] and was the first study of its kind to speciate *M. bovis*, *M. californicum*, and *M. bovigenitalium*, taking ~4 hours to complete the assay. In addition, this study demonstrated that the 3 newly developed probe assays were more accurate than partial 16S rRNA gene sequencing used as the gold standard for comparison; 4 samples that were identified by 16S rRNA gene sequencing as *M. bovis* only were found to contain *M. bovis* and *M. bovigenitalium* by the probe PCR assays (confirmed by subsequent amplicon sequencing).

The use of real-time multiplex PCR assays has also been explored allowing the simultaneous detection of *M. bovis*, *M. californicum*, and *M. bovigenitalium* in a single reaction.[61] Specificity testing showed appropriate amplification for all *M. bovis*, *M. californicum*, and *M. bovigenitalium* isolates tested, with no amplification against other Mollicutes and Eubacterial isolates tested. For milk, bull semen, and swab (vaginal, preputial, nose, and eye) samples, a 10^1 to $10^3 \times$ cfu/mL increase in the limit of detection was found when all 3 target species were present in

TABLE 1 Overview of the characteristics of the PCR methods described for use in diagnosing Mycoplasma species in cattle

	Conventional PCR	Real time PCR SYBR green dye	Real time PCR fluorescent reporter probe
Overview	Measures the amount of PCR product at the end of the PCR, therefore requires gel electrophoresis to visualize amplified DNA	Measures PCR amplification as the PCR occurs	Measures PCR amplification as the PCR occurs
	Is semiquantitative through comparison of gel band intensities	Is quantitative	Is quantitative
		Dye binds to all double stranded DNA. As the quantity of target double stranded DNA increases, the amount of light emitted by the dye increases proportionally.	Probe binds to a specific targeted region internal to the primer binding sites. As the PCR cycles progress, the reporter dye is cleaved off and fluoresces. An increase in PCR product targeted by the probe causes a proportional increase in fluorescence.
		Increase in background noise because of dye binding to all double stranded DNA	Commercial Assays available
Specificity	Often targeted 16S rRNA gene, therefore cross-amplification seen between *M. bovis* and *M. agalactiae*[33]	Melt dissociation curve can be used to differentiate between species or nonspecific amplification[47]	Alternative target genes and the use of a specific internal fluorescent probe allowed for greater specificity[20,21,49–51,61]
	Can differentiate amongst *Mycoplasma* spp. based on gel banding patterns after product digest step,[40] or product size on a gel[44]		Allows multiplexing to detect multiple targets in a single reaction
Sensitivity	$\sim 10^2$-10^3 cfu/mL in milk[44]	Less commonly used to detect mycoplasma in cattle; sensitivity not reported	$\sim 10^1$ cfu/mL in milk samples for *M. bovis, M. californicum*, and *M. bovigenitalium*[49]
			$\sim 10^2$ cfu/mL in lung samples for *M. bovis*[50]
			$\sim 10^5$, 10^4, and 10^7 cfu/mL in semen samples, and $\sim 10^6$, 10^4, and 10^7 cfu/mL in swab samples for *M. bovis, M. californicum*, and *M. bovigenitalium*, respectively[61]

Abbreviation: PCR, polymerase chain reaction.

the sample at the same time to simulate a multispecies infection. When examining field samples, the real-time PCR and culture followed by 16S-23S rRNA sequencing produced similar results; however, the PCR was able to identify 5 samples with a multispecies infection.

Commercial real-time PCR assays targeting *Mycoplasma* species are also available, providing results within 4 hours. This includes (but is not limited to) several PathoProof assays by Thermo Fisher Scientific (Scoresby, Victoria, Australia), several Mastit4 assays by DNA Diagnostic A/S (Risskov, Denmark), the bactotype Mastitis HP3 PCR kit by QIAGEN Pty Ltd (Chadstone Centre, Victoria, Australia), and the Bovicheck *M. bovis* PCR kit by Biovet Inc. (Quebec, Canada). DNA Diagnostic A/S also offers a Pneumo4 assay, which allows the identification of *M. bovis* in tracheal lavage and deep nasal swabs. Analyses of the PathoProof PCR assay for the detection of *M. bovis* has estimated a herd-level specificity of 97%-100%.[62] The PathoProof Major-3 kit (Thermo Fisher Scientific) has been used for latent class analysis of BTM samples for herd-level diagnosis of *M. bovis* in Danish herds.[63] The Mastit4B assay (DNA Diagnostics A/S) has been used to determine within-herd prevalence of *M. bovis* intramammary infection and its association with milk yield, somatic cell count and milk composition in an Estonian dairy herd.[64] To the best of our knowledge, the above kits by QIAGEN and Biovet Inc are yet to be used in any published studies. While these assays provide commercially available PCR options, their use is targeted to milk

samples only (with the exception of Pneumo4), and published literature involving their use in the field is limited.

3.3 | Overview of current PCR methods

Table 1 provides an overview of the characteristics of the PCR methods described for use in diagnosing *Mycoplasma* species in cattle. Because of the importance of *M. bovis* in the dairy industry, the focus has been on developing PCR assays targeting *M. bovis* in milk samples. Few studies have investigated PCR assays on other sample types and their limit of detection, or for other important *Mycoplasma* species in the dairy industry. Given that mycoplasma infections are not limited to mastitis, it is important that other sample types such as joint fluid aspirates and mucosal surface swabs are further investigated. While *M. bovis* is currently considered the most important mycoplasma pathogen in cattle, there are several other species, which can cause significant disease, including *M. californicum, M. leachii, M. dispar, M. canadense*, and *M. alkalescens*, which should not be overlooked.

3.4 | Isolate typing

Several tools have been used for genetic characterization of *M. bovis* isolates such as pulsed field gel electrophoresis (PFGE), amplified

fragment length polymorphism (AFLP), multiple locus variable number tandem repeat analysis (MLVA), and multilocus sequence typing (MLST). Pulsed field gel electrophoresis was developed in the early 1980s and has been widely used for mycoplasma strain typing and identification. During PFGE, chromosomal DNA is first digested across a small number of sites along the chromosome using a restriction enzyme, resulting in large DNA fragments being separated in an agarose gel producing band patterns specific to different strains.[65] Pulsed field gel electrophoresis has been used in several *Mycoplasma* outbreak investigations.[66,67] An early study examining *M. bovis* and *M. californicum* isolates from numerous body sites of cattle associated with a single herd outbreak found that isolates from the mammary gland frequently had identical PFGE patterns to isolates from other body sites, suggesting internal transmission of a single strain.[66] A second study investigating an *M. bovis* outbreak across 6 cattle feedlots suggested, through identical PFGE patterns of 39 isolates, that *M. bovis* strains follow a clonal epidemiological spread at the herd-level and the same strain can persist in calves within the herd after clinical signs have disappeared.[67]

Amplified fragment length polymorphism is a PCR-based technique also used for DNA fingerprinting, which involves digestion of the DNA with restriction enzymes, ligation of adaptors, and selective amplification of a subset of restriction fragments followed by gel analysis of these fragments.[68] This technique was used to analyze genetic variation among 42 *M. bovis* field isolates from Danish cattle over a 17 year period from 1981 to 1998 to monitor the genetic relatedness of isolates over time.[69] This was done by identifying the likely sources of infection and transmission of the pathogen, as well as the possible role the host, environment, and pathogen play in disease outcome. Despite Mollicutes being considered to be quite dynamic in their evolution,[70] the study found remarkable genetic homogeneity amongst Danish *M. bovis* isolates, which were likely to be epidemiologically related, and had remained genetically stable for a considerable amount of time. The study also observed identical genetic patterns amongst *M. bovis* isolates from lung, nasal, and milk samples from a single animal, which was consistent with the findings by Biddle et al,[66] suggesting internal spread of the pathogen. It also suggests that the site of isolation may not be associated with differences in the genetic profile of the organism, corresponding with the phenotypic findings by Thomas et al[71] who demonstrated no correlation between the pathological background of *M. bovis* isolates (mastitis, pneumonia, and arthritis), and their ability to adhere to different host cell lines.

Multilocus sequence typing uses several genes within the genome, which are analyzed for unique sequences.[65] Based on these differences the relatedness of the isolates is determined. Multiple locus variable number tandem repeat analysis uses PCR to amplify regions of DNA, which contain repeated sequences, which tend to be unstable and, as such, can be used to distinguish different isolates based on the amount of repeats at selected loci.[65] For genotyping *M. bovis* isolates, it has been recommended that both methods are used, with MLST used first to characterize strains followed by MLVA for fine scale typing.[72] In a recent study, MLVA and MLST were used to analyze 60 *M. bovis* isolates collected in France over a 35 year period (from 1977 to 2012) to

determine population diversity.[73] Based on 4 loci, the study found that all isolates fell into 2 separate clusters: those collected before the year 2000 and those collected after the year 2000. Interestingly, recent strains showed more homogeneity than older strains, which is consistent with the spread of a single clone, however is contradictory to the Danish findings that found more heterogeneity was evident amongst more recent isolates.[69] The author of the French study hypothesizes that the loss of heterogeneity in the recent French strains may be because of selection of multiresistant clones.

In a recent global study by Rosales et al,[74] MLST was used to characterize 137 *M. bovis* isolates from healthy and clinically infected animals from 12 different countries including Australia and countries located in Europe and Asia. Using a set of 7 housekeeping genes, MLST analysis identified 2 distinct population clusters. Cluster one included most of the British and German isolates, while cluster two contained European, Asian, and Australian isolates, which were more geographically distant and heterogeneous. This supports the hypothesis that once *M. bovis* is introduced into a country, most likely through trade, the pathogen undergoes geographically independent evolution. This is clearly demonstrated with Australian, Chinese, and some Israeli isolates exhibiting genetic diversity in comparison to most of the European isolates characterized. The study also noted that while most of the mastitis isolates clustered largely with the pneumonia isolates, the 7 most genetically diverse isolates, which did not cluster were all mastitis cases, with the author suggesting this may be because of adaptation to the specific niche ecology of the bovine mammary gland. This is despite previous studies suggesting that the site of isolation may not be associated with differences in the genetic profile of the organism.[66,69,71] However with little information available regarding the circumstances surrounding their isolation, there may be other aspects associated with these isolates, which attributed to the genetic diversity amongst this group. Similar findings were presented by Menghwar et al[75] who also used an MLST scheme to compare *M. bovis* isolates from China, Israel, and Australia. Isolates from these 3 countries clustered together as a single dominant clone, which was the same sequence type as identified by Rosales et al.[74]

While typing methods for *Mycoplasma* spp. other than *M. bovis* are scarce, the use of PFGE and MLVA has been demonstrated on *M. californicum* strains isolated from bovine milk samples in Japan between 2005 and 2013.[76] These methods were able to demonstrate sufficient discriminatory power to allow epidemiological analysis, and as such may be the basis for future investigations into *M. californicum* outbreak and transmission events, particularly in countries where this species is more prevalent. Further research is needed into genotyping methods for other important *Mycoplasma* spp. in cattle.

3.5 | Whole genome sequencing

Whole genome sequencing (WGS) is becoming a widely used tool for investigating bacterial genome sequences as high throughput sequencing becomes faster, cheaper, and more readily available. As suggested by the name, WGS involves sequencing the entire genome of selected isolates which can then be used for clinical diagnostics, disease

outbreak investigation and controlling antimicrobial resistance.[77] To date, the complete genome sequences are available for 5 *M. bovis* isolates[78–82] and 2 *M. californicum* isolates,[83,84] as well as a single *M. arginini*,[85] *M. bovigenitalium*,[86] *M. canadense*,[87] *M. bovoculi*,[88] and *M. leachii* isolate.[89] While the complete sequencing of these isolates has provided some insight into the content and dynamics of the organism as well as uncovering putative virulent genes, few studies have used WGS to compare the genetic diversity between a large number of isolates. A recent study examined 82 Australian *M. bovis* isolates collected over a 9 year period (2006–2015) from various geographical locations and bovine anatomical sites.[90] Through SNP analysis it was suggested that a single strain existed, with comparative genomics revealing minimal variation between isolates from different anatomical locations, again consistent with previous findings using PFGE[66] and AFLP.[69] Furthermore, minimal variation was also observed between isolates from clinically infected and subclinical carrier animals, an area not previously investigated. This marked genomic similarity observed amongst an array of isolates obtained from different clinical backgrounds, suggests that the disease outcome may be determined largely by host and environmental factors, with pathogen factors playing a less significant role.

4 | SEROLOGICAL DIAGNOSIS

4.1 | *M. bovis* antibody detection in plasma, serum, and individual milk samples

While culture and PCR diagnosis relies upon demonstrating the presence of the mycoplasma organism, indirect ELISAs are available that demonstrate the presence of anti-*Mycoplasma* antibodies in plasma, serum, and milk samples. Their purpose is to identify animals that have been exposed to the pathogen, and had time to mount a humoral immunological response. Because of *M. bovis* currently being considered the most common and important species, much of the assay development and research available is specific to this species. This includes the commercially available indirect ELISA kits for anti-*M. bovis* antibody detection by Bio-X Diagnostics (Rochefort, Belgium) for use on serum and milk samples, which has been used in several field and assay validation studies.[63,91–95] The Bovichek *M. bovis* antibody ELISA test kit by Biovet Inc (Quebec, Canada) is also commercially available for use on serum samples and has been used in studies evaluating in-house assays.[95,96]

When developing an ELISA assay, the potential for cross-reactivity of an *M. bovis* specific ELISA with other bacterial species found in cattle and ruminants is difficult to assess but is important from a diagnostic perspective, particularly if these species are not of clinical importance. Several studies developing *M. bovis* ELISA assays for use on bovine serum have addressed this issue by aligning the sequence of the *M. bovis* protein being used with other mycoplasma and bovine pathogens through online databases such as NCBI to identify the theoretical cross-reaction potential.[96–98] In vitro analysis using western blot has also been used to investigate the presence of reactive proteins in other pathogens of interest.[98,99] Western blot is often used in concurrence with sequence alignment to provide both an in vitro analysis and theoretical analysis of the potential for cross-reactivity of an ELISA.[100] However, cross-reactivity experienced in-field is difficult to determine, and as such care must be taken when interpreting results.

An indirect ELISA assessing the immune response of animals to *M. bovis* can prove to be a complementary tool, which overcomes some of the challenges of culture and PCR analysis. While culture and PCR rely on the animal shedding the organism at the time of sampling, an ELISA can measure past exposure to the organism in the form of a humoral antibody response which is usually sustained for a period of time after infection and therefore does not require detection of an organism that may be shed intermittently or is no longer present. As a result, when these diagnostic methods are used concurrently there is often a much higher prevalence of *M. bovis* positive animals detected by ELISA than by PCR or culture.[101,102] However, the presence of anti-*M. bovis* antibodies does not indicate an animal is necessarily harboring or shedding viable *M. bovis*. This was demonstrated in an *M. bovis* mastitis outbreak scenario where all clinically affected cows were culled from the herd; however, all remaining cows (with the exception of 3) were ELISA positive despite being free of disease.[103] Once the clinically affected animals were removed, the herd recovered from its outbreak with the only evidence of previous infection being ELISA positive animals remaining. In an unpublished study, 45.8% of bulls (n = 118) were ELISA positive post joining after use in 4 herds with a recent or current *M. bovis* herd infection, however only 3.8% of these bulls were culture positive for *M. bovis* in semen samples, and none displayed any clinical signs of disease (Hazelton MS, Sheehy PA, Bosward KL, et al. Subclinical Mycoplasma infections in bulls before and after introduction into *Mycoplasma bovis* infected dairy herds. Unpublished manuscript: The University of Sydney, Sydney School of Veterinary Science, Faculty of Science; 2017). Lastly, in a study involving the examination of 3 age groups (110, 310, and 510 days old) of male cattle for *M. bovis* on a breeding station, a higher percentage of animals were identified as positive by ELISA in serum samples (75, 50, and 55%) than by culture from samples taken from the nasal cavity (52, 30, and 27%).[104] These studies demonstrate that while the number of animals exposed to *M. bovis* may be high, the proportion of animals that succumb to disease or are actively shedding the pathogen is frequently much lower. Furthermore, it suggests that culling of animals based on ELISA results alone is not a logical approach towards controlling an outbreak and will likely result in over culling. Therefore, ELISA results should be interpreted in conjunction with other diagnostic approaches.

While an antibody ELISA may not be affected by intermittent shedding of *M. bovis*, one diagnostic challenge can be false negative results because of inadequate time for seroconversion to occur, as well as a lack of antibody persistence. Several trials involving experimental infection have demonstrated that seroconversion takes 2–3 weeks post exposure.[97] In a study involving 16 clinically affected cows identified to be PCR positive for *M. bovis* in milk samples after natural infection, 15 were ELISA positive in sera 7–13 days after the time of diagnosis of each cow. Based on the recommended cut-off value for the ELISA, 3 of these 15 cows were marginally positive, highlighting that time since exposure is an important factor influencing ELISA results. However, after a disease outbreak, it is unlikely that all animals

TABLE 2 Strengths and limitations of mycoplasma diagnostic methods

	Culture	PCR	Antibody ELISA
Strengths	Inexpensive (costs may vary between countries and laboratories)	Organism does not have to be viable as it targets the DNA of the organism	Measures antibody response, therefore animal does not need to be shedding the organisms at the time of sample collection
	Can detect most *Mycoplasma* species[19]	Quick diagnosis turnaround of several hours[31]	Only blood or milk sample required to assess immune response
		Can discriminate between different *Mycoplasma* spp.[40,49]	Longevity of antibody expression is possibly several months[103,114]
		Can discriminate *Mycoplasma* spp. from *Acholeplasma* spp.[30]	
Limitations	Fastidious growth requirements[19]	Higher cost	
	Diagnosis turnaround of up to 10 days[10]	Many mycoplasma PCRs are species specific, therefore eliminating the detection of other species[21,51]	Uncertainty around cross-reactivity with other organisms
	Unable to discriminate between *Mycoplasma* spp. and *Acholeplasma* spp., which may lead to false positives[19]	Animal must be shedding the organism at the time the sample was taken[26,28]	Seroconversion may take 2–3 weeks before antibodies can be detected[97]
	Unable to discriminate between different *Mycoplasma* species[19]	Identification of non-viable organisms may lead to insignificant positive results	Suggestions of poor sensitivity[63,115]
	Organism must be viable, therefore storage and handling of the sample is important[7,11]		
	Animal must be shedding the organism at the time of sampling[26,28]		

Abbreviation: PCR, polymerase chain reaction.

will become exposed at the same time. These differences in exposure time are likely to result in differences in ELISA titers across the herd, as demonstrated by Le et al.[105] In this study involving 103 animals from herds involved in *M. bovis* outbreaks, serum was collected within 2 months of the onset of clinical disease, with ELISA titers showing a broad and scattered distribution. Therefore, the progression of pathogen exposure within a herd must be considered when deciding on when to sample a herd and how to interpret the results.

Regarding antibody longevity, a study involving a small dairy herd found 94% of animals remaining in the herd were positive for *M. bovis* antibodies in sera 27 weeks after an *M. bovis* outbreak began, and 15 weeks after the last clinical case was observed.[103] However, while antibody persistence in sera may be quite extended, this may not be the case in milk samples. In the same study, composite milk samples collected from the same animals just 2 weeks before the sera samples (25th week post outbreak) revealed only 2% of the animals were positive for *M. bovis* antibodies in milk.[103] Furthermore, in individual quarter milk samples collected from 6 animals with clinical *M. bovis* mastitis, all quarters from all animals were positive for *M. bovis* antibodies at the 9th week post outbreak; however, at the 20th week only 4 of these animals were antibody positive in the previously clinically infected quarters. This suggests that *M. bovis* antibody levels are likely to be much lower or possibly undetectable in milk samples as compared with serum samples and this may be because of the way serum antibodies infiltrate into the mammary gland. In milk whey, there are significantly lower concentrations of immunoglobulins compared to serum.[106] Some immunoglobulins including IgM and IgA are produced locally in the mammary gland, however IgG, which is often targeted by an ELISA, is

produced in the blood and transferred into the milk.[107] Therefore, it is not surprising that *M. bovis* antibodies detected by ELISA are higher in sera compared with milk samples. Milk immunoglobulin concentration can also be affected by production factors with a significant increase in milk IgG_1 concentrations as lactation number increases.[108] A decrease in milk IgG_1, IgG_2, IgM, and IgA concentrations can also be seen as the number of days in lactation increases, with an increase in concentration again in the late stages of lactation coinciding with a drop in milk production.[108,109] Milk immunoglobulin levels have also been shown to increase during udder inflammation.[107,110] When interpreting results, it is therefore important to take into account that milk samples may produce lower than expected readings compared with serum, and this will be dependent on the clinical history of *M. bovis* infection, lactation number, stage of lactation, and current health status in regards to mastitis.

4.2 | *M. bovis* antibody detection in bulk tank milk samples

An alternative application of the ELISA is for detection of exposure to *M. bovis* at a herd-level via BTM analysis. This is applicable to assessing biosecurity risks when purchasing animals from a herd of unknown *M. bovis* status. Because of collection ease and its representation of the lactating herd, a BTM sample is commonly used for PCR or culture analysis as a surveillance tool to measure the herd-level prevalence of *Mycoplasma* spp. across different regions and countries.[47,111–113] Recent studies have evaluated the use of the Bio-X Diagnostics anti-*M. bovis* antibody ELISA kit on BTM samples to estimate herd-level

prevalence.[63,91,93] It has been suggested that the percentage optical density coefficient (%ODC) cut-off recommend for the kit may be too low when used on BTM, and as such, should be increased from 37% to 50% ODC to achieve fewer false positives without compromising the negative predictive value. This modification does however, decrease the sensitivity from 60.4% to 43.5%.[63] As with individual serum samples, discrepancies have also been found between PCR and ELISA BTM results, with significantly higher proportions of samples being positive by ELISA than by PCR.[63,91] Yet given that the tests are detecting separate aspects of the disease, presence of the pathogen (detected with the use of PCR) and the herd immune response to the pathogen (analyzed with ELISA), this is not unexpected and an important biological aspect to consider when interpreting test results.

When considering using BTM for antibody detection, it is also important to have an understanding of the factors that may be associated with its variation. A study by Petersen et al[93] demonstrated that the anti-*M. bovis* antibody positive prevalence of lactating cows was significantly associated with the BTM ELISA %ODC in Danish dairy herds, yet prevalence of antibody-positive young stock and herd size was not. While a significant association was also found by Parker et al[91] in Australian dairy herds, within-herd seroprevalence explained little of the variation in the BTM ELISA %ODC. Time since the initial *M. bovis* outbreak within a herd and time since the onset of the calving period were found to significantly affect the BTM ELISA %ODC.[91] The same study found the BTM ELISA %ODC to be significantly higher in samples collected from the hospital herd compared with the lactating herd. This is likely because there is a higher chance of animals being exposed to *M. bovis* in the hospital herd compared with the lactating herd because of common disease management practices. While several questions remain regarding the use of an indirect-ELISA for *M. bovis* diagnostics, the literature suggests that when analyzing BTM samples, ELISA and PCR are most useful in combination as they provide complimentary diagnostic information on the infection status of a herd from different biological perspectives.

5 | SUMMARY

Mycoplasmas can cause serious disease in cattle herds resulting in significant negative economic and welfare impacts. Rapid diagnosis facilitates prompt disease control and prevention. In the face of a disease outbreak there are often several diagnostic questions relating to the source and distribution of infection within and across herds. Although slow to achieve a result, culture provides a definitive isolate that can be used for DNA extraction and typing to investigate the source of infection and relatedness of isolates to other strains of the organism. Polymerase chain reaction assays provide rapid diagnostic results. Expeditious diagnostics facilitate timely decision making regarding clinically affected cattle providing opportunity for management to mitigate the risk of disease transmission. Serology provides an option for assessing the recent exposure of different herds or specific cohorts of animals within herds. The diagnostic approach pursued needs to consider budgetary constraints, urgency for diagnosis, test availability and sample type and handling conditions. While no single test is without disadvantages because of their limitations, their combined use will complement each other based on their individual strengths (Table 2).

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

ORCID

Alysia M. Parker http://orcid.org/0000-0002-6122-8645

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