

Research Article

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Diagnostic performance of a novel molecular microarray assay for detection of bovine intramammary infections with *Mycoplasma* species using a gold standard approach

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Abstract

Diagnosis of cases of *Mycoplasma* mastitis is particularly challenging due to their unique biological characteristics, which complicate diagnosis and treatment. Hence, accurate and quick diagnostic tests for early detection of *Mycoplasma* mastitis are essential to initiate appropriate interventions or culling. The objective of this research is to estimate the diagnostic performance of the molecular microarray assay (MMA) against bacterial culture for the diagnosis of bovine intramammary infections (IMI) with *Mycoplasma* spp., using a gold standard approach and the Kappa agreement coefficient. A total of 395 quarter milk samples were collected from cows in 31 dairy herds with conventional milking systems in California, USA. Following dairy personnel practices, milk samples were collected from the lactating cows showing abnormal milk characteristics and shipped within 24 hours to the laboratory for bacterial culture and MMA examination. Milk samples with positive growth were confirmed via PCR test to eliminate misdiagnosis of *Acholeplasma* spp. Eighty-seven cows (22%) were positive for *Mycoplasma* spp. IMI and the test accuracy was 88.4%. The sensitivity of MMA was 90.8% (95% CI (Confidence Interval): 82.68–95.95), and the specificity was 87.66% (95% CI: 83.46–91.12). The positive predictive value of MMA in these herds was 67.52% (95% CI: 60.51–73.83), and the negative predictive value was 97.12% (95% CI: 94.57–98.49). Calculated Kappa coefficient was 0.70 (95% CI: 0.618–0.778). The high estimates of sensitivity and specificity of MMA suggest its usefulness as a routine and quick test for accurate diagnosis of *Mycoplasma* spp. IMI in dairy cows. Our findings indicate that MMA holds promise for enhancing the detection of *Mycoplasma* spp. and could potentially revolutionize diagnostic practices in the dairy industry and supports udder health management.

Mastitis, inflammation of the mammary gland, significantly affects milk production and quality, leading to substantial economic losses and increased veterinary costs (Bradley, 2002). *Mycoplasma* species are emerging as critical pathogens in the dairy industry, primarily due to their role in causing mastitis in dairy cows. Among the various etiological agents of mastitis, *Mycoplasma* spp. are particularly challenging due to their unique biological characteristics, which complicate diagnosis and treatment (Nicholas *et al.*, 2008). In 2021, it was reported that *Mycoplasma* spp. were the most commonly isolated pathogen in the bulk tank milk samples from United States dairy herds between 2016 and 2019 by 96.2% (Gioia *et al.*, 2021). Additionally, Kusiluka *et al.* (2006) found that *M. bovis* was associated with up to 52% of *Mycoplasma* mastitis in California, USA, whereas Gourlay *et al.* (1989) concluded that up to 37% of calves' mortality due to respiratory tract disease in the UK was caused by *M. bovis*. In the United States, the overall losses due to mastitis caused by *M. bovis* were estimated at USD 108 million per year (Rosengarten and Citti, 1999; Okella *et al.*, 2023).

Monitoring udder health and detecting infected cows early is impossible without reliable and affordable diagnostic methods (Lam *et al.*, 2009). Accurate diagnostic tests for early detection of pathogen-specific mastitis are essential to initiate the appropriate interventions or culling (Britten, 2012). Early detection of contagious *Mycoplasma* mastitis is particularly important to initiate the appropriate interventions or culling and avoid the within-herd infectious transmission. Traditional diagnostic methods for *Mycoplasma* infections have predominantly relied on bacteriological culture as an important component of a farm control strategy to minimize a herd

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outbreak (Okella *et al.*, 2023). This technique involves isolating the pathogen from milk samples using specialized media and incubating them under controlled conditions. Although culture methods are considered the gold standard due to their high specificity, they come with notable challenges. Bacterial culture is labour-intensive, time-consuming and requires several days to weeks for definitive results (Schukken *et al.*, 2013). The culturing of fastidious *Mycoplasma* species can be lengthy and require specialized media and incubation conditions, which may result in delayed or false-negative results that can increase the response time of a farm to a *Mycoplasma* outbreak (Nicholas *et al.*, 2008; Gioia *et al.*, 2016). The low bacterial load in subclinical cases and the contamination by other faster-growing bacteria can hinder the detection of *Mycoplasma* spp. through culture, leading to false negatives or delayed diagnosis (Spergser *et al.*, 2007; Nicholas *et al.*, 2008).

The limitations of culture methods have driven interest in alternative diagnostic techniques. The molecular microarray assay (MMA) has proven to be a highly useful tool for the diagnosis of mastitis in dairy cows and the detection of mastitis-causing pathogens (Gioia *et al.*, 2018). This technology allows for the simultaneous detection and identification of multiple pathogens in a single sample, offering a significant advantage over traditional culture methods, which are often time-consuming and limited in their ability to detect fastidious or mixed infections. By using species-specific DNA probes, microarray assays can accurately identify key mastitis pathogens, including *Staphylococcus aureus*, *Escherichia coli* and various species of *Streptococcus* and *Mycoplasma*, even in subclinical cases (Koskinen *et al.*, 2009). Additionally, microarray assays provide quicker results, enabling timely intervention and more targeted antimicrobial treatments, which can reduce the overall use of antibiotics and improve animal welfare and milk production (Verbeke *et al.*, 2014). The adoption of MMA could address several of the limitations associated with traditional culture techniques, such as prolonged turnaround times and the need for specialized laboratory conditions.

To the best of our knowledge, the performance of the MMA against established culture methods in the context of dairy cow milk diagnostics has not been extensively studied. Therefore, a comprehensive evaluation of MMA compared to traditional culture methods is crucial to understanding their relative benefits and limitations. The objective of this research is to fill this gap by estimating the diagnostic performance of MMA against conventional culture methods for the diagnosis of *Mycoplasma* species in dairy cow milk using the gold standard approach. The results could provide valuable insights into the feasibility of integrating MMA diagnostics into routine udder health management, ultimately contributing to effective management of *Mycoplasma* infections and improved herd health.

Materials and methods

Study population and sample collection

A total of 434 quarter milk samples were collected from different cows in 31 dairy herds with a conventional milking system in the San Joaquin Valley, CA, south of Fresno. Milk samples were collected from the lactating cows showing abnormal milk characteristics, such as the presence of flakes, clots or watery milk.

The cows were separated by the farmer and restrained in headlocks during sampling. Dairy personnel provided initial screening, identifying animals with abnormal milk during milking. Each herd was visited by trained Valley Veterinarians, Inc. (VVI) personnel

who trained the dairy personnel on the proper procedures for collecting milk samples aseptically. Quarter foremilk samples were collected aseptically from the selected cows for bacterial culture examination and microarray assay testing. After the farm personnel had carried out their routine pre-milking practices and before attachment of the milking unit, quarter milk samples were collected according to NMC (1999) guidelines. About 10 ml milk was collected aseptically from each quarter of each cow in clean sterile snap-cap plastic tubes, which were placed in ice boxes at 5°C and were delivered to the Laboratory of VVI (California, USA) within 48 hours of collection.

Bacteriological culturing

All milk samples were cultured as recommended by the National Mastitis Council (NMC, 1999) for isolation of *Mycoplasma*. *Mycoplasma* culture was performed using 10 µl of milk samples of pooled quarters inoculated onto BBA plates and Myco plates acquired by VVI from Udderhealth Systems. Samples are plated using an autoclaved cotton swab that is dipped into the sample after thawing, swabbed into 1/6th of a BBA plate, then the swab is rotated before swabbing into 1/8th of a Myco plate. Plates were read at 3 and 7 d by a mastitis laboratory technician with several years of experience, possessing the ability to distinguish colonies likely to be *Mycoplasma* spp. Plates with colonies that were morphologically consistent with those of *Mycoplasma* spp. were classified as positive. Additionally, samples with positive growth were confirmed via PCR test to eliminate misdiagnosis of *Acholeplasma*. Results were considered positive if any *Mycoplasma* colonies were seen and negative if there was no evidence of growth of *Mycoplasma* spp. Samples with one colony or more (≥ 1 CFU = colony forming unit) were considered positive for *Mycoplasma* species. If the sample was defined as contaminated and the *Mycoplasma* species was part of such contamination, the sample was considered positive.

Molecular microarray assay

The procedures of MMA include the following steps: 50 µl of milk is transferred to a 96 deep well plate, and surfactant is added. The plate is spun down at 6000× g for 5 min, and the supernatant is removed, leaving the bacterial pellet behind. The pellet is washed with a buffer, and the supernatant is removed. The pellet is treated with lysis buffer, liberating the DNA. A novel variant of PCR called the Asymmetric Exponential and Linear Amplification (AELA), which generates large numbers of single-stranded amplicons, was used according to Banda *et al.* (2022). In brief, AELA-PCR introduces an amplification strategy that makes use of both exponential and linear amplification of the target nucleic acid. This is done by specifically designed primers and the choice of adequate thermal profiles. A Cy5-labelled primer is used. The PCR amplified DNA is combined with the array hybridization buffer and transferred to a well on the 96-well immunoassay plate. Each well contains one copy of the microarray. The 96-well plate is placed on the heater-shaker plate and exposed to elevated DNA denaturing temperature, followed by a hybridization temperature. The well is imaged in a plate scanner. The image processing utilized a proprietary algorithm, where the image intensities are converted to a semi-quantitative scale: Scant, +, ++, +++, +++++, ++++++, where ++++++ is approximately 500,000 CFU/ml. Samples indicated as 'scant' were labelled as positive for MMA.

Table 1. Cross-tabulation of bacterial culture (gold standard) and the new test, molecular microarray assay (MMA) for detection of *Mycoplasma* species in milk samples

Test	Bacterial culture positive	Bacterial culture negative	Total
MMA test positive	79 (A)	38 (B)	117
MMA test negative	8 (C)	270 (D)	278
Total	87	308	395

Statistical analysis

A traditional gold standard method was employed for the evaluation of the diagnostic performance of MMA, where bacterial culture served as the gold standard/reference test. The collected data were screened for unlikely or missing values before carrying out any valid statistical analysis. Subsequently, a descriptive statistical analysis was performed for both the test results of MMA and bacterial culture tests. A contingency table containing information on the proportion/ frequency of true positive (TP), true negative (TN), false positive (FP) and false-negative (FN) was generated by comparing the MMA and culture tests. The diagnostic sensitivity, diagnostic specificity, positive predictive values (PPV), and negative predictive values (NPV) were estimated. The PPV and NPV were calculated at a different prior probability of infection. Additionally, to assess the level of agreement between the results of the MMA and bacterial culture, we calculated the Kappa coefficient using Epitools (<https://epitools.ausvet.com.au/>).

Results

A total of 434 milk samples were collected from 31 herds for bacterial culture and MMA testing. Due to high somatic cell count and elevated DNA contents in the MMA testing, 30 samples were inhibited and excluded from the analysis. In addition, nine samples where *Mycoplasma* spp. was not part of the contamination were regarded as contaminated on the bacterial culture (having three different types of colonies). A total of 395 samples with clean and complete observations were submitted to perform the statistical analysis. The results of cross-tabulation between bacterial culture that is combined with PCR test confirmation as the gold standard and the MMA as the new diagnostic test for detection of *Mycoplasma* spp. are presented in Table 1. The number of TP, TN, FP and FN cases is shown in cells A, D, B and C, respectively.

Based on the numbers presented in Table 1, the sensitivity of MMA was 91% (95% CI: 82.68–95.95), the specificity of MMA was 88% (95% CI: 83.46–91.12), the PPV of MMA was 68% (95% CI: 60.51–73.83) and the NPV of MMA was 97% (95% CI: 94.57–98.49). The estimated prevalence was 22%, whereas the test accuracy was 88.4%. At a prior probability of infection of 0.5, the positive predictive value of MMA detection is 88%, which reflects the probability that cows with a positive test (MMA detection) do truly have the infection (*Mycoplasma* spp. positive). The negative predictive value was 91%, which means the probability that cows with a negative test (MMA detection) truly do not have the disease (*Mycoplasma* spp. negative) (Chikere *et al.* 2019). The high NPV of the MMA test supports the use of this test for screening purposes. The calculated Kappa coefficient to quantify the agreement between the two tests was 0.70 (95% CI: 0.618–0.778). According

to the Kappa scale (Landis and Koch, 1977), this result indicates a 'substantial agreement; Kappa between 0.61 and 0.80' between the MMA and bacterial culture for the diagnosis of *Mycoplasma* spp. in milk samples.

Discussion

Mycoplasma infections are a significant cause of mastitis, often leading to severe economic losses in the dairy industry. The improved diagnostic accuracy is critical for timely intervention and proper herd management, as *Mycoplasma* mastitis can spread rapidly through a herd if not properly identified and controlled. Traditional culture methods can be challenging due to the slow-growing and fastidious nature of *Mycoplasma* species, which can result in false negatives or delayed diagnosis (Fox *et al.*, 2005). It is challenging to implement timely responses in cows with positive culture results of *Mycoplasma* species due to the highly contagious nature of this pathogen and the long culture period (Okella *et al.*, 2023). This delay may lead to the rapid transmission of the disease to other animals within the herd. MMA allows for the rapid and accurate detection of *Mycoplasma* spp. by targeting species-specific DNA sequences, which offer a substantial improvement in diagnostic accuracy over traditional methods.

Our findings reflected that MMA showed good diagnostic sensitivity and specificity estimates compared to the bacterial culture for the diagnosis of *Mycoplasma* spp. from milk samples with IMI. By directly targeting the DNA of *Mycoplasma* spp., MMA can bypass the need for bacterial growth, thereby significantly reducing the time to diagnosis. Previous studies have shown that molecular assays can provide results within hours, as opposed to the days or weeks required for bacterial culture (Parker *et al.*, 2018). In cases of *Mycoplasma* mastitis, this rapid detection is particularly important because *Mycoplasma* infections can spread quickly through a herd, leading to widespread economic losses if not promptly managed.

The sensitivity of MMA is a key factor in their superior diagnostic performance. Sensitivity refers to the assay's ability to correctly identify infected cows (i.e., true positives). Since molecular methods detect the pathogen's genetic material, they are capable of identifying even very low levels of *Mycoplasma* DNA in milk samples, which would likely be undetectable by culture or require special media or a longer period for confirmation. This is particularly relevant in subclinical or early-stage infections, where the bacterial load may be too low to be detected by traditional methods (Koskinen *et al.*, 2009). In such cases, early detection facilitated by MMA can allow for timely intervention, reducing the risk of transmission and progression of the disease.

MMA has the capacity to detect multiple *Mycoplasma* spp. simultaneously, this is a significant advantage over culture methods, which can only identify the *Mycoplasma* at the genus level (NMC, 1990). Different *Mycoplasma* species can cause varying degrees of mastitis severity, and being able to identify the specific species present in a herd, MMA can guide more targeted and effective treatment strategies (Al-Farha *et al.*, 2017). With bacterial culture, the delayed or inaccurate identification of *Mycoplasma* spp. can lead to the inappropriate use of antibiotics, as veterinarians may resort to broad-spectrum treatments while awaiting diagnostic results. By providing faster and more accurate diagnoses, MMA can support the more judicious use of antimicrobials, helping to reduce the risk of antimicrobial resistance in dairy herds (Nicholas *et al.*, 2008). This is particularly relevant for *Mycoplasma* infections,

as they are often resistant to many commonly used antibiotics, and inappropriate treatment can worsen the disease situation (Gelgie *et al.*, 2022, 2024).

Specificity, on the other hand, refers to the assay's ability to correctly identify animals that are not infected (i.e., true negatives). MMAs are designed to target species-specific genetic markers, which minimises the likelihood of cross-reactivity with other organisms present in the milk. This is especially important when dealing with *Mycoplasma* spp., as mixed infections are common in dairy herds, and other bacterial species may grow more readily in culture, potentially leading to misdiagnosis. Culture methods are unable to differentiate between closely related species. For example, *Mycoplasma* spp. cannot be distinguished from *Acholeplasma*, a species considered non-pathogenic, on a modified Hayflick medium without additional tests (Okella *et al.*, 2023). Parker *et al.* (2018) reported that differentiation between *Mycoplasma* spp. and *Acholeplasma* spp., an environmental contaminant, by bacterial culture alone can result in a false *Mycoplasma* positive sample. In contrast, molecular assays provide a highly specific diagnosis by distinguishing *Mycoplasma* from other mastitis pathogens (Verbeke *et al.*, 2014). This high level of specificity reduces the risk of false positives, ensuring that treatment decisions are based on accurate pathogen identification. Furthermore, MMA has the capacity to detect multiple *Mycoplasma* spp. simultaneously, which is a significant advantage over culture methods. Different *Mycoplasma* spp. can cause varying degrees of severity in mastitis, and being able to identify the specific species present in a herd can guide more targeted and effective treatment strategies (Al-Farha *et al.*, 2017). For instance, *M. bovis* is often associated with more severe chronic infections and knowing its presence allows veterinarians to implement stricter biosecurity and control measures to prevent further spread.

Studies have shown that molecular methods outperform bacterial culture, particularly in subclinical cases or when the bacterial load is low. For example, Koskinen *et al.* (2009) reported that molecular assays had a higher detection rate of *Mycoplasma* in milk samples compared to conventional culturing techniques. This improved diagnostic accuracy is critical for timely intervention and proper herd management, as *Mycoplasma* mastitis can spread rapidly through a herd if not properly identified and controlled. Consequently, the use of MMA in routine diagnostics can lead to better mastitis management, reduced economic losses, and more appropriate antibiotic use, as it provides reliable, quicker, and more comprehensive results than traditional methods.

Future studies should consider the application of Bayesian latent class analysis instead of gold standards as an ideal approach for diagnostic test evaluation of *Mycoplasma* spp. in dairy cows, where none of the tests under evaluation is regarded as a perfect test.

Conclusions

MMA offers substantial advantages over bacterial culture for diagnosing IMI with *Mycoplasma* species in dairy cows. The sensitivity and specificity of the MMA were high, 91% and 88%, respectively, suggesting its usefulness as a routine test for accurate and rapid diagnosis of *Mycoplasma* species IMI in dairy cows. MMA holds promise for enhancing the detection of *Mycoplasma* species and could potentially revolutionize diagnostic practices in the dairy industry and improve udder health management.

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Competing interests. Our co-authors, Nicolaas Smit and Holger Klapproth, who are affiliated with Safeguard Biosystems Holding Ltd, the developer of the molecular microarray assay, plan to introduce a product named 'DairyGuard' based on this technology to the veterinary diagnostic market. We confirm that the company had no impact on the data handling, statistical analysis, or presented contents in this paper.

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