TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Short Communication

Turk J Vet Anim Sci
(2020) 44: 752-755
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doi:10.3906/vet-1811-62

# Application of an indirect MilA ELISA for the detection of Mycoplasma bovis antibodies in bovine milk

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**Received:** 24.11.2018 • **Accepted/Published Online:** 03.03.2020 • **Final Version:** 02.06.2020

**Abstract**: The objective of this study was to detect Mycoplasma bovis specific antibodies using a recently reported MilA ELISA with the aim to detect M. bovis antibodies in milk. An indirect ELISA, based on a recombinant fragment of the Mycoplasma immunogenic lipase A (MilA) protein, was conducted on 291 milk samples for the detection of M. bovis antibodies. Samples were also tested with conventional Mycoplasma culture and M. bovis PCR. Samples were collected from individual cows from 2 commercial dairy herds in South Australia. Of 291 samples tested, 68 (23.4%) were detected positive for M. bovis antibodies, 150 (51.5%) were positive for M. bovis in PCR and 166 (57.0%) in bacterial culture. These results indicate that MilA indirect ELISA can be utilized for the detection of M. bovis antibodies in milk.

**Key words:** Mycoplasma, antibodies, mastitis, ELISA, PCR, MilA

Mycoplasma bovis is increasingly raising concerns in the dairy industry as a mastitis associated pathogen. It can cause significant economic losses as a result of a decrease in milk production, an increase in somatic cell counts (SCC), decreasing product value, the cost of associated treatments [1], and implementation of eradication strategies [2]. As a result, early and accurate detection of M. bovis in milk has attracted the attention of recent research, to better improve the strategic control of the disease [3]. Cattle subclinically infected with M. bovis, otherwise known as "carrier cows," pose significant challenges, particularly if introduced to an uninfected herd as newly purchased stock [4,5]. Among these challenges, for instance, is the recognition of carrier cows at the time of sampling.

The difficulties in detection of M. bovis from milk samples via culture or Polymerase Chain Reaction (PCR) is commonly attributed to the intermittent shedding of the pathogen in milk [6], or the inability of current diagnostic tests to identify the pathogen during the convalescent phase of the disease. Hence, identifying a diagnostic test capable of accurately detecting M. bovis in milk samples from carrier cows is necessary.

Indeed, some immunogenic proteins from M. bovis have been evaluated previously for their capacity in detection of M. bovis antibodies [7,8]. Other commercial ELISA kits have been used for M. bovis antibodies screening at bulk tank milk levels in Danish and Australian dairy herds [5,9,10]. However, the recombinant Mycoplasma immunogenic lipase (MilA) Immunoglobulin G (IgG) indirect ELISA has not been evaluated in milk. It has been developed and evaluated in experimentally infected calves with M. bovis using serum [11]. MilA has shown greater sensitivity and comparable specificity to other commercial ELISA kits (BIO K302 and BIO K260 from BioX Diagnostics (Belgium)). Therefore, it has been recommended for serological screening for M. bovis in

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cattle [11,12]. As a result, this study aimed to detect M. bovis antibodies with the MilA ELISAs in milk samples and compare these results to the presence or absence of M. bovis in the samples, as determined by conventional bacterial culture and PCR methodologies.

A total of 291 milk samples were collected aseptically from individual cow once from 2 commercial dairy farms in South Australia. Of these, 251 were collected from Farm 1, a 2500-cow dairy located in the south east region of South Australia and were previously used in another study [13]. The remaining 40 samples were collected from Farm 2, a 400-cow dairy located in the mid-north region of South Australia. Both farms had a history of repeated mastitis treatment failure and high SCC, however no clinical signs of M. bovis diseases were observed at the time of sampling for any of the sampled cows. The problem of high SCC has been historic on both farms, M. bovis has previously been detected on Farm 1 (using a commercial laboratory for testing of milk for presence of M. bovis) but there was no previous detection of M. bovis on Farm 2.

All 291 samples were analyzed by MilA ELISA, bacterial culture and PCR. The MilA ELISA was conducted to test for M. bovis antibodies in milk following the procedure described previously [12]. The ELISA cutoff value was calculated using Bayesian latent class modelling in multiple populations [11] and was estimated at 140 antibody units (AU) (therefore all tests above this value were considered as positive). All samples were also subjected to purified glutathione-S-transferase (GST) ELISA.

For bacterial culture analysis, an aliquot of each milk sample (250 µL) was added to a Mycoplasma broth (Oxoid, Australia) and left for 5 days before plating on Mycoplasma media (Oxoid, Australia). Plates were incubated for 15 days at 37° C using 10% $CO_2$ jars. The plates were then examined for colonies using a stereomicroscope (Olympus SZ30, Australia). Samples were considered positive when at least a single Mycoplasma colony was detected [14]. To confirm isolation of Mycoplasma, 3–5 selected colonies from each plate were subcultured into the enriched Mycoplasma broth and inoculated under the same conditions and checked for color change of broth and typical Mycoplasma colonies on agar. As soon as the phenol red indicator changed to yellow, subcultures into fresh broth and onto agar were carried out.

DNA extraction was performed directly on 250 µL from each milk sample (Qiagene, Germany), and a DNA concentration measurement was performed using Nanodrop 1000c (Thermofisher Scientific Inc., Waltham, MA, USA). HRM-real time PCR was conducted on each of these samples according to [15] using M. bovis specific primers targeting 16S rRNA, including primer forward: 5'-CCAGCTCACCCTTATACATGAGCGC-3' and primer reverse: 5'-TGACTCACCAATTAGACCGACTATTTCACC-3' [16]. Following recommendation by Behera et al. [17], the gene copy number cutoff value detected by our qPCR was estimated at 30 copies.

Results of the ELISA indicated that 68/291 samples (23.4%) were positive for M. bovis antibodies (≥140 AU). By farm, 63/251 (25.1%) and 5/40 (12.5%) samples were positive from Farms 1 and 2, respectively. In comparison, a total of 166/291 samples (57.0%) were positive for Mycoplasma like colonies by conventional culture. By farm, 144/251 (57.4%) and 22/40 (55.0%) samples were positive from Farms 1 and 2, respectively. Finally, 150/291 (51.5%) samples were positive for M. bovis by PCR, all of which were from Farm 1 (Table).

Another previously described ELISA, designed with similar conditions using GST protein as coating antigen, was also carried out to ensure the signals in MilA ELISA were due to the M. bovis antigen and not the result of the GST fusion antigen. All samples tested in the MilA ELISA negative to GST protein (data not shown).

A total of 24/68 (35.3%) and 23/68 (33.8%) samples were positive by indirect ELISA and negative on bacterial culture and PCR, respectively (Table). This may have occurred due to several reasons. Firstly, indirect ELISAs not only detect animals undergoing current infections but also those which have undergone past exposure to the pathogen; and secondly, the sensitivity of the indirect ELISA to detect antibodies during the convalescent phase of the disease when the cow has seroconverted and cleared the organism.

Another reason for positive ELISA detection versus negative culture and PCR can be explained by the intermittent shedding of the pathogen through milk [18]. Moreover, in advanced stages of M. bovis infection, the impermeability of mammary gland epithelium to the antibodies detected by ELISA could be another reason for the discrepancies between detection of antigen and antibody [19]. In this study, PCR had higher rate of positive samples than ELISA. This is not an unusual detection. ELISA detects antibodies and PCR detect the antigen. One possible explanation for the high discrepancy may be the incubation or prodromal period of the majority of sampled cows when antibodies are not present yet, but cows are shedding the antigen found by the PCR. Impermeable mammary gland may result in prevention of seroconversion that may have resulted in the low prevalence of antibodies in milk. After recovery, the persistence of the antibodies to M. bovis in the mammary gland may be short, resulting in a transient detection of antibodies in milk only. Not all media are suitable for testing of antibodies by ELISA. Milk may be unsuitable media, and this needs further research. It is also possible that the cutoff used in the MilA ELISA was too high for bovine milk, as the cutoff used in this study

**Table.** Count of Mycoplasma bovis samples per outcome of indirect ELISA (140AU) versus both culture and qPCR results of 291 samples collected aseptically from 2 farms in South Australia.

| ELISA results (no.) | Bacterial culture results (no.) | | | | | | | PCR results (no.) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Positive | | | Negative | | | Total | Positive | | | Negative | | | Total |
| | Farm 1 | Farm 2 | Total | Farm 1 | Farm 2 | Total | | Farm 1 | Farm 2 | Total | Farm 1 | Farm 2 | Total | |
| Positive | 43 | 1 | 44 | 20 | 4 | 24 | 68 | 45 | 0 | 45 | 18 | 5 | 23 | 68 |
| Negative | 101 | 21 | 122 | 87 | 14 | 101 | 223 | 105 | 0 | 105 | 83 | 35 | 118 | 223 |
| Total | 144 | 22 | 166 | 107 | 18 | 125 | 291 | 150 | 0 | 150 | 101 | 40 | 141 | 291 |

had not been the same as one used previously on bovine serum samples [11]. Testing the sensitivity and specificity at different ELISA cutoffs indicated that the cutoff value for ELISA at 140 was the recommended threshold; however, it did give a low sensitivity (30.0%) but relatively good specificity (83.7%). It should be noted that the PCR was probably not the appropriate 'gold standard' test for testing the prevalence of M. bovis in milk and this should be an area of a future research.

This is the first study where the MilA ELISA has been used to detect M. bovis specific antibodies in bovine milk. The results indicate that this ELISA could be utilized for improving biosecurity on dairy farms, not for detecting positive cows (low sensitivity) but for confirming positive cows as they were likely true positive (high specificity). The application of the MilA ELISA to detect antibodies in milk may need further validation compared to commercial ELISA kits approved for use on milk. The cutoff value for the MilA ELISA may need to be reevaluated for bovine milk and this would require further studies on farms with known M. bovis outbreaks. Indeed, it would be more beneficial to repeatedly test cows with known timing of infection (e.g., by challenge model) and estimate the timing of appearance and waning of the antibodies in bovine milk.

**Acknowledgments**

The authors would like to express their special thanks to the farmer and farm staff for their cooperation and interest in the study. Abd Al-Bar Al-Farha was partially funded by the Higher Committee for Education Development in Iraq in the form of a scholarship. All field and laboratory procedures were financially supported by the University of Adelaide.

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