Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds

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ABSTRACT

The relevance and limitations for using measurements of antibodies against *Mycoplasma bovis* in bulk tank milk (BTM) as a potentially cost-effective diagnostic tool for herd classification has not been evaluated before. Assuming that an increasing or high seroprevalence is a result of on-going or recent spread of *M. bovis* in a dairy herd, we tested the hypothesis that increasing prevalence of antibody-positive cows and young stock are associated with increasing BTM antibody ELISA values against *M. bovis* in Danish dairy herds with different courses of *M. bovis* infection. Furthermore, we tested whether herd size was associated with variations in the BTM responses. Thirty-nine Danish dairy herds selected to represent 4 different herd-level infection groups [8 control herds, 14 acute outbreak herds, 7 herds with previous outbreaks, and 10 herds with elevated BTM ELISA-values directed against *M. bovis* (>64% optical density measurement)] were visited 4 to 5 times, approximately 3 mo apart. At each visit, 65 young stock were blood sampled. At the milk recording date closest to the herd visit date, 50 milk recording samples from individual lactating cows were randomly selected. In addition, a BTM sample was collected as a representative sample directly from the bulk tank by the dairies' milk truck drivers as part of the mandatory milk quality-control scheme. Blood and milk samples were tested for antibodies against *M. bovis* with a commercially available ELISA test (Bio-X BIO K 302, Bio-X Diagnostics, Rochefort, Belgium). A linear mixed effects model was used to analyze the effects of the prevalence of antibody-positive lactating cows and young stock and herd size on the BTM *M. bovis* ELISA results. Herd was included as a random

effect to account for clustering of BTM samples originating from the same herd. Increasing prevalence of antibody-positive lactating cows was the only variable associated with increasing *M. bovis* BTM ELISA optical density measurement. In contrast, the prevalence of antibody-positive young stock did not correlate with the BTM optical density measurement. In conclusion, some *M. bovis* associated herd infections are detectable by BTM ELISA-testing, but limitations exist and further investigations of the effect of different clinical disease expressions in the herds are warranted.

Key words: *Mycoplasma bovis*, ELISA, bulk tank milk, antibody

INTRODUCTION

Mycoplasma bovis can cause severe disease and production losses in both dairy and beef cattle herds. In adult cattle, *M. bovis* infection is often associated with mastitis, but arthritis and pneumonia can also be seen. In calves, the typical disease manifestations are otitis media, pneumonia, or arthritis (Maunsell et al., 2011). *Mycoplasma bovis* seems to be an emerging pathogen in countries all over the world, and even though *M. bovis* was first isolated in Denmark in 1981 (Friis, 1984), it was not considered a major pathogen in Danish cattle before 2011. However, the Danish cattle industry has had increased focus on this infection over the last couple of years due to an increase in the number of severe outbreaks of *M. bovis*-associated disease on the herd level.

Traditionally *M. bovis* has been detected by bacteriological culture (BC) from either individual milk samples or bulk tank milk (BTM) samples. In recent years detection by PCR has become more widely used, as it is less time consuming and apparently can produce similar sensitivity and specificity to conventional BC methods (Pinnow et al., 2001; Cai et al., 2005). At the individual level, antibodies directed against *M. bovis* can be detected in serum and milk 1 to 2 wk after uptake of the bacteria (Boothby et al., 1987; Byrne

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et al., 2005), but the use for diagnosis in individual animals is not always straightforward (Maunsell et al., 2011). *Mycoplasma bovis* can also be isolated from asymptomatic carrier animals (Punyapornwithaya et al., 2010), but it is not known how the antibody response in these animals reacts compared with clinically ill animals. However, in beef cattle, group-level antibody titers and seroconversion can be associated with active infection (Martin et al., 1990), and spread of the disease in a dairy herd could therefore be expected to lead to a marked increase in seroprevalence. Except for Nielsen et al. (2015), who evaluated the performance of an antibody-detecting ELISA against PCR for BTM for national screening purposes, the use of antibodies in BTM for diagnosing either a disease or the presence of *M. bovis* in specific dairy herds has not been addressed in published literature. Antibody measurements on BTM have been used as a diagnostic tool for the control of other infectious diseases because they can be easy and inexpensive to use in national surveillance programs (Lindberg and Alenius, 1999; Nielsen, 2013). To use antibodies against *M. bovis* in BTM for surveillance purposes, however, it is essential to know which factors influence the antibody level in BTM.

The use of ELISA on BTM samples to classify or monitor dairy herds for *M. bovis* infection will, in a setting such as Denmark, be of interest, as the sampling can be automated via a mandatory milk quality-control scheme and is inexpensive compared with BC and PCR. A requirement for BTM antibody testing to be useful is that there must be a good correlation between the BTM antibody level and the prevalence of infection in individual cattle in the herd. In the case of other infectious diseases, such as *Salmonella* Dublin, bovine virus diarrhea virus, and Q-fever, it has been shown that the level of antibodies in the BTM correlates well with the within-herd prevalence of antibody-positive cows (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et al., 2012). Increasing herd size has been shown to be a risk factor for presence of *M. bovis* infection (Thomas et al., 1981; Pinho et al., 2013). On the other hand, antibodies might be diluted in herds with a large number of cows contributing milk to the BTM (Nekouei et al., 2015). Hence, herd size may have to be taken into account when evaluating BTM testing for herd diagnosis.

The objective of our study was to test the hypothesis that increasing within-herd prevalence of antibody-positive lactating cows and increasing seroprevalence in young stock increases the BTM antibody ELISA values against *M. bovis* in Danish dairy herds. Furthermore, we wanted to test whether herd size affected the level of antibodies in BTM.

MATERIALS AND METHODS

Populations

The target population was all Danish dairy herds enrolled in the voluntary milk recording system (https://www.landbrugsinfo.dk/Kvaeg/RYS/Sider/RYS_English.aspx), which at the beginning of the study period consisted of approximately 3,000 (90% of all) Danish dairy herds. Their average annual milk yield per cow was 9,663 kg of milk and the average herd size was 166 lactating cows. The study population consisted of herds about which the Knowledge Centre for Agriculture (now SEGES, Aarhus, Denmark) had prior knowledge about *M. bovis*-associated diseases either from farmers or veterinarians. SEGES is the merger of the former Knowledge Centre for Agriculture and the Danish Pig Research Centre, effective as of January 1, 2015. The company is owned by the farmers and provides knowledge, consultancy, and technology to all Danish farmers (<https://www.seges.dk/en>).

Only herds with more than 100 dairy cows were included. More than 100 cows were needed to make sure the herd had enough young stock to sample. The study population consisted of 39 dairy herds selected by a veterinarian at SEGES during the period from March 2013 to February 2014. The veterinarian at SEGES had prior knowledge about the herds from national screenings in 2012 and 2013, where BTM from all dairy herds were tested for antibodies against *M. bovis* and with PCR, as well as information provided by the local consulting veterinarian in the herds. To ensure collection of data from herds with different severity and duration of disease, the following criteria were used to select herds to fit into 1 of 4 groups before enrollment in the field data collection part of the study.

- Control herds: negative in diagnostic tests (PCR, ELISA, and BC), no history of clinical signs that could be related to *M. bovis* over the past 3 years; 8 herds.
- Case herds-acute: Recent clinical suspicion of disease associated with *M. bovis*; 14 herds. In these 14 herds, the presence of *M. bovis* was confirmed by positive *M. bovis* PCR [PathoProof, Thermo Fisher Scientific, Waltham, MA; with a cycle threshold (Ct) <37] in milk samples from individual cows or BTM. In 4 herds the confirmation was performed in BTM, in 5 herds at individual cow level, and in 5 herds at both individual cow level and in BTM. In addition, 5 of the 14 herds were positive for *M. bovis* in BC of samples from individual animals.

Figure 1. Distribution of bulk tank milk (BTM) ELISA optical density measurements (ODC%) of antibodies against *Mycoplasma bovis* in herds initially selected as control herds, case herds with acute outbreaks, case herds with previous outbreak, and case herds with high BTM. The lines connect results from the same herd.

- Case herds-previous: Previous clinical suspicion of disease associated with *M. bovis*; 7 herds. This group included herds with former *M. bovis* test-positive clinically ill animals, but that no longer had any acutely diseased animals. In these 7 herds, the presence of *M. bovis* was confirmed by positive PCR [PathoProof PCR (Ct <37)] in milk samples from individual cows and BTM in 3 herds, and in BTM in 4 herds.
- Case herds-BTM: high ELISA values against *M. bovis* in BTM (Bio-X Bio K 302, Bio-X Diagnostics, Rochefort, Belgium; ELISA value >64% optical density measurement) in a national screening in summer 2013; 10 herds.

The selection of farms was done as described above to ensure representation of all types of clinical signs, infection, and test patterns in the study herds so that the full scale of BTM and seroprevalences were represented in the data set for analysis. The allocation to groups was not used in the analyses.

The distribution of BTM optical density (ODC%) measurements from the different herds over time were divided into the 4 categories as shown in Figure 1. We

aimed to include herds of different sizes and geographical locations; however, systematic stratification according to these factors was not used. More than 90% of the Danish dairy cattle are located on the peninsula of Jutland, and all herds enrolled in our study were located in Jutland. Because the prevalence of *M. bovis* infection is low, the selection criteria were used to ensure inclusion of herds with evidence of disease or spread of *M. bovis*.

Each herd was visited 4 to 5 times, approximately 3 mo apart. At each visit, 65 young stock equally distributed in the age group 0 to 12 mo old were blood sampled. At the milk recording date closest to the herd visit date, 50 milk recording samples from individual lactating cows were randomly selected. A BTM was sampled as a representative sample while the bulk tank was emptied by the dairies' milk truck drivers as part of the mandatory milk quality-control scheme.

Detection of Antibodies

Milk samples from both individual animals and BTM and serum samples from the young stock were analyzed for antibodies against *M. bovis* using the commercial kit Bio-X BIO K 302 *Mycoplasma bovis* ELISA kit at

Eurofins-Steins Laboratory (Holstebro and Vejlen, Denmark). A sample coefficient was calculated as: $ODC\% = (OD \text{ sample} - OD \text{ negative control}) / (OD \text{ positive control} - OD \text{ negative control}) \times 100\%$, where OD is the optical density measured by the ELISA reader for each test sample, and negative and positive control samples on the sample ELISA plate. For animal-level testing, a sample coefficient ≥ 37 ODC% was considered positive, and a sample coefficient < 37 ODC% was considered negative according to the recommendations of the manufacturer of the ELISA kit. To our knowledge the test has not been evaluated with regard to sensitivity (**Se**) and specificity (**Sp**) for animal-level diagnosis in the field. It has been evaluated for use on BTM in national screening of dairy herds for national or regional prevalence estimation by Nielsen et al. (2015). The Se and Sp at cut-off 37 ODC% were 60.4 and 97.3, respectively. At a cut-off of 50 ODC%, the Se was 43.5 and the Sp was 99.6.

Description of Variables

The outcome variable was the continuous *M. bovis* BTM ODC%. Four explanatory variables were tested as potential explanatory variables of the *M. bovis* BTM ODC%.

- The apparent prevalence of antibody-positive lactating cows: calculated as the proportion of cows with individual-ELISA ODC% ≥ 37 in milk out of all tested cows in the herd on the sampling day.
- The apparent prevalence of antibody-positive young stock: calculated as the proportion of young stock with individual-ELISA ODC% ≥ 37 in blood out of all tested young stock in the herd on the sampling day.
- The apparent prevalence of antibody-positive lactating cows > 50 ODC%: to assess if an effect existed of the ELISA cut-off used for apparent prevalence calculations, the apparent prevalence was also calculated as the proportion of cows with individual-ELISA ODC% > 50 (**ELISA50**) in milk.

- Herd size: calculated as the average number of cows in the herd, in the quarter of the year where the BTM sample was collected.

An observation was excluded if it was not possible to match the date of the apparent prevalence with a BTM sample within ± 30 d or if the number of animals for the prevalence calculations was low ($n < 30$).

Statistical Analysis

Scatter plots of all the explanatory variables plotted against each other were assessed to evaluate whether linear relationships existed between the variables. Variables which were highly correlated ($\rho > 0.8$) were not included in the same model.

Two linear mixed effects models were created. The models were built by backward stepwise elimination of nonsignificant variables and their 2-way interactions. The criteria for keeping a variable in the model was $P < 0.05$, and the model fit was assessed by Akaike’s information criteria (**AIC**); a lower AIC led to a better model. The *P*-values were calculated as an ANOVA comparison between a model with all variables and a model without the specific variable and its interaction terms.

Herd was included as a random effect to account for clustering of BTM samples originating from the same herd. The explanatory degree of the model was assessed by calculation of the ratio: $(R_e - R_{fm} / R_e)$, where R_e is the residual variance of the model only containing the random effect of herd and R_{fm} is the residual variance of the final model. Data management and analyses were made using “R: A language and environment for statistical computing” version 3.0.2 (www.r-project.org).

RESULTS

Descriptive Statistics

Data selection yielded 113 observations distributed on 37 herds with 2 to 5 observations per herd, with 3 observations, on average, per herd. Descriptive statistics

Table 1. Descriptive statistics of bulk tank milk (BTM) *Mycoplasma bovis* ELISA optical density measurement (ODC%) and the explanatory variables tested in models for BTM *M. bovis* ELISA ODC% in 37 herds (113 observations)

Item	Minimum	Quartile 1	Median	Quartile 3	Maximum
BTM <i>M. bovis</i> ELISA ODC%	6	19	26	36	87
Prevalence of antibody-positive lactating cows (≥ 37 ODC%)	0	0.04	0.1	0.18	0.77
Prevalence of antibody-positive lactating cows (> 50 ODC%)	0	0.02	0.05	0.1	0.49
Herd size	76	201	273	367	779

Figure 2. Descriptive statistics showing the bulk tank milk *Mycoplasma bovis* ELISA optical density measurement (ODC%) plotted against the apparent prevalence of antibody-positive lactating cows.

of the outcome, *M. bovis* BTM ODC%, and explanatory variables are shown in Table 1. A visual presentation of the raw data is provided in Figure 2, where the BTM ELISA ODC% is plotted against the apparent prevalence of antibody-positive lactating cows.

When adding the prevalence of antibody-positive young stock to the data set, many observations were lost when limiting the prevalence calculation to ± 30 d from the BTM date. Therefore, another data set was created that only contained the prevalence of antibody-positive young stock and the BTM samples closest to the date of the prevalence calculation ($n = 116$). Descriptive statistics of the young stock prevalence are shown in Table 2. From Figure 3 it is apparent that the prevalence of antibody-positive young stock did not correlate well with the BTM *M. bovis* ELISA ODC%,

and the variable was therefore not included in further analysis.

Analytical Statistics

Collinearity was found between the apparent prevalence of antibody-positive lactating cow and ELISA50, which were consequently not tested simultaneously, but with the same explanatory variables in different models.

The resulting final model included only the apparent prevalence of antibody-positive lactating cows. The model had the AIC closest to 0 and showed the best prediction when evaluating the plots of predicted versus observed values visually. The final model explained 54% of the variation (Table 3).

Figure 3. Bulk tank milk *Mycoplasma bovis* ELISA optical density measurement (ODC%) plotted against the apparent prevalence of antibody-positive young stock.

Table 2. Descriptive statistics of the prevalence of *Mycoplasma bovis* antibody positive young stock [≥ 37 optical density measurement (ODC%)] in 39 herds (116 observations)

Clinical sign	Minimum	Quartile 1	Median	Quartile 3	Maximum
Prevalence of antibody-positive young stock (≥ 37 ODC%)	0.00	0.12	0.28	0.38	0.66

The predicted *M. bovis* ELISA ODC% in BTM is plotted against the observed values in Figure 4. Overall, the model predicted the BTM values well, even though a tendency toward overestimation of the high values and underestimation of the low BTM values may have occurred.

DISCUSSION

Our objective was to test the associations of different factors with the variation in BTM antibodies against *M. bovis* in Danish dairy herds. We found that a rather large proportion of the variation could be explained by the apparent prevalence of antibody-positive lactating cows.

The prevalence of antibody-positive lactating cows was positively associated with the BTM ODC%. Each time the prevalence increased by 10%, the BTM ODC% increased by 9 ODC%. This means that as the number of antibody-positive cows in the herd increases, indicative of recent spread of *M. bovis* bacteria, we can expect the BTM ODC% to increase. This association is in agreement with other studies on other infectious diseases in dairy herds (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et al., 2012). For *Salmonella* Dublin, Nielsen and Ersbøll (2005) also found that the degree of explanation increased when including the prevalence or number of high ELISA responders and whether or not the herd had had a positive BC for *Salmonella* Dublin. In our study, the prevalence of high ELISA responders could not be included in the same model as the prevalence, and unfortunately we did not have sufficient BC results for *M. bovis* from all farms or comprehensive and consistent systematic recordings of clinical disease associated with *M. bovis* in individual animals. These findings would have been interesting to study regarding their effects.

Even though the prevalence of antibody-positive cows is associated with the BTM ODC%, it is more ambiguous than seen with other diseases. In our data set and according to our final model, the prevalence of antibody-positive cows was above 30% before the BTM on average went above the cut-off of 37 ODC% (Table 3 and Figure 2), indicating that a large proportion of the cows had to have been exposed to *M. bovis* to make the BTM antibody testing able to detect it with reasonably Se and Sp (Nielsen et al., 2015). This hampers the ability to classify herds based on a BTM sample. A more persistent pattern has been found for *Coxiella burnetii* measurements in BTM, where all samples above the cut-off value had a within-herd prevalence of at least 20% (Muskens et al., 2011). The discrepancy may arise because many *M. bovis* clinically diseased and medically treated cows do not contribute to the bulk tank. The apparent prevalence in our study stems from samples from individual cows at milk recording. Most of these cows would have contributed to the BTM on the day they were sampled. A minor part of medically treated cows could also have been part of milk recording, but the milk from those cows would not have entered the BTM due to procedures for preventing antibiotic residues entering the milk for consumption.

As mentioned in the introduction, the use of antibodies to detect disease among individual animals is not straightforward, and clinical disease is not always followed by a rise in antibodies (Maunsell et al., 2011). Unfortunately, evaluation of antibody reactions in individual animals in field studies is sparse. On the group level, however, antibody titers show correlation with disease in beef cattle (Martin et al., 1990), which would suggest that the same could be the case for dairy herds. A lack of investigations of the correlation between antibodies in milk and serum in the literature also exists, but the manufacturer of the used

Table 3. Results of the final model describing explanatory variables and random effects of bulk tank milk (BTM) ELISA optical density measurement (ODC%) for *Mycoplasma bovis*

Variables (explains 54% of the variation)	Variance	SD	Estimate	SE	P-value
Random effects					
Herd	19	4			
Residuals	80	9			
Fixed effects					
BTM ELISA ODC% (intercept)			17	1.4	—
Prevalence of antibody-positive lactating cows (per 10% increase)			9	0.7	<0.001

Figure 4. Predicted bulk tank milk *Mycoplasma bovis* ELISA optical density measurement (ODC%) values plotted against observed values for the model ($n = 113$). The line shows the regression line between observed and predicted values.

ELISA test states in a datasheet about the test that the correlation is 0.59 (<http://www.biox.com/Default.aspx?tabid=64&utid=215>). In an unpublished field study (L. Nielsen, University of Copenhagen, Frederiksberg, Denmark) from Denmark, 1,442 paired serum and milk samples from 8 dairy herds had a correlation of 0.7. When considering the different clinical manifestations of *M. bovis* disease, it could be that antibodies in milk are not a good measure of ongoing disease in a dairy herd. A better understanding of the correlations between different clinical signs, excretion of bacteria, and serum and milk antibodies would help interpret the BTM antibody response.

Herd size was not associated with the BTM ODC% in herds in our study. Other studies have found an increasing probability of isolating *M. bovis* by BC from the BTM with an increased number of lactating cows (Thomas et al., 1981; Pinho et al., 2013). This is probably related to the different outcomes in the studies, and the fact that in our model the presence of *M. bovis* is already taken into account by the within-herd prevalence. Our study investigated the factors associated with variance in BTM ODC%, whereas other studies have investigated risk factors for a BC-positive BTM. With increasing herd size a risk exists that the contribution of antibodies to the BTM by 1 cow becomes diluted (Nekouei et al. 2015). For *Salmonella* Dublin, a better explanation of the BTM ODC% was found when using the mean yield-corrected ODC%, also indicating a dilution effect in the BTM (Nielsen and Ersbøll, 2005); this was not the case in our study.

As mentioned, *M. bovis* can give rise to a variety of clinical signs in different age groups; thus, a BTM sample may or may not be able to detect all types of disease manifestations in a herd. Two questions arise

from this: (1) is it possible to detect disease among young stock in the BTM, and (2) is it possible to detect all types of disease manifestations among cows in the BTM. We included the prevalence of antibody-positive young stock as an explanatory variable to partially clarify this issue. The prevalence of antibody-positive young stock did not correlate with the BTM ELISA ODC%, indicating that the status of young stock is not reflected in the BTM. Hence, to determine the status of the young stock, samples from individual animals are probably needed. Further studies on this matter are definitely warranted.

The other part of this question is whether or not disease among cows manifested primarily as arthritis, for example, will be detectable in a BTM sample. Unfortunately, we do not have systematically recorded information about the prevalence of the different disease manifestations in the different herds, so this issue cannot be further elucidated in our study. Further studies where the distinction in the expression of clinical disease can be made are warranted.

Another model with the prevalence of lactating cows based on ELISA50 as the explanatory variable instead of the prevalence at the recommended cut-off at 37 ODC% was tried. This did not change the model fit when the other explanatory variables were the same (results not shown). The reason for exploring the effect of changing the cut-off is that there is a lack of evidence for the optimal ELISA cut-off at animal level with regard to detection of infected or infectious animals within infected herds. A higher cut-off might detect more truly infected animals as opposed to previously exposed animals; hence, the ELISA50 prevalence might be better correlated with the BTM antibody level. However, this did not seem to be the case. We did not

try high cut-off values because few cows had higher ELISA-responses.

To the best of our knowledge, no studies have evaluated antibodies in BTM as a diagnostic tool for *M. bovis* in relation to the underlying disease manifestation in dairy herds. Nielsen et al. (2015) evaluated the overall performance of the BTM test method for national or regional screening purposes and provided estimates of Se, Sp, and predictive values. However, the estimates were associated with much uncertainty due to few test-positive herds in the data set. The results of Nielsen et al. (2015) and the present study complement each other. Our study illustrates that the lack of Se may be due to the fact that quite high prevalence of affected animals are required for the BTM antibody level to increase. As discussed, the results from our study are in overall agreement with similar studies about other infectious diseases, such as *Salmonella* Dublin, bovine viral diarrhoea virus, and *Coxiella burnetii* infections (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et al., 2012). However, we also found some challenges that have to be addressed to use BTM-ELISA testing as a tool in herd level *M. bovis* diagnosis of dairy herds.

In most instances, the prevalence estimates were not based on the same date of sampling, but within ± 30 d of the BTM sample. Hence, we cannot be certain that milk from all the individual cows used for calculating the prevalence was present in the BTM sample. To evaluate the limitation of this, a data set consisting of 87 of the observations (75%) sampled within ± 14 d of the BTM sample were used to rerun the final model. This rerun model yielded approximately the same estimates as the model based on the larger data set, and did not make the predictions for the model better. Hence, our final model appeared to be robust to the uncertainties in the prevalence estimation related to the time of BTM sampling. In individual animals the antibody response can persist for at least 6 mo (Nicholas et al. 2002). Nonetheless, from our data it seems to be important to realize that the BTM antibody level is actually quite dynamic, and a high response in BTM does not necessarily persist for long time (Figure 1).

The repeated measurements in theory have a temporal structure, but this was ignored and a simple random effect used because any temporal effects from such a small number of repeated measurements were considered to be uninteresting and to have a small effect on the data. In addition, our primary interest was not to describe the nature of the dependency between the BTM measurements, so the random effect was merely included to take potential dependencies into account in order not to overestimate the effect of the explanatory variables in the final model.

CONCLUSIONS

Our objective was to identify factors that influence the variation in BTM ELISA ODC% against *M. bovis* in Danish dairy herds. Increasing prevalence of antibody-positive cows was associated with increasing *M. bovis* BTM ELISA ODC%. In contrast, the prevalence of antibody-positive young stock did not correlate with the BTM ODC%. Herd size was not associated with *M. bovis* BTM ELISA ODC%. A combination with distinction between different clinical signs would be very interesting, but the available data did not support such an investigation. More studies to investigate risk factors for variance in BTM ELISA ODC% for *M. bovis* and potential combinations of test procedures to use for herd classifications are warranted before this method can be deemed useful for disease-control purposes.

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REFERENCES

- Boothby, J. T., D. E. Jasper, and C. B. Thomas. 1987. Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on local and systemic antibody response. *Can. J. Vet. Res.* 51:121–125.
- Byrne, W., B. Markey, R. McCormack, J. Egan, H. Ball, and K. Sachse. 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. *Vet. Rec.* 156:767–771.
- Cai, H. Y., P. Bell-Rogers, L. Parker, and J. F. Prescott. 2005. Development of a real-time PCR for detection of *Mycoplasma bovis* in bovine milk and lung samples. *J. Vet. Diagn. Invest.* 17:537–545.
- Friis, N. F. 1984. *Mycoplasma bovis*-induced mastitis in cattle in Denmark. *Nord. Vet. Med.* 36:324–325.
- Lindberg, A. L. E., and S. Alenius. 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. *Vet. Microbiol.* 64:197–222.
- Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. *Can. J. Vet. Res.* 54:337–342.

- Maunsell, F. P., A. R. Woolums, D. Francoz, R. F. Rosenbusch, D. L. Step, D. J. Wilson, and E. D. Janzen. 2011. *Mycoplasma bovis* infections in cattle. *J. Vet. Intern. Med.* 25:772–783.
- Muskens, J., E. v. Engelen, C. v. Maanen, C. Bartels, and T. J. G. M. Lam. 2011. Prevalence of *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. *Vet. Rec.* 168:79.
- Nekouei, O., H. Stryhn, J. VanLeeuwen, D. Kelton, P. Hanna, and G. Keefe. 2015. Predicting within-herd prevalence of infection with bovine leukemia virus using bulk-tank milk antibody levels. *Prev. Vet. Med.* 122:53–60.
- Nicholas, R. A. J., R. D. Ayling, and L. P. Stipkovits. 2002. An experimental vaccine for calf pneumonia by *Mycoplasma bovis*: clinical, culture, serological and pathological findings. *Vaccine* 20:3569–3575.
- Nielsen, L. R. 2013. Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle. *Vet. Microbiol.* 162:1–9.
- Nielsen, L. R., and A. K. Ersbøll. 2005. Factors associated with variation in bulk-tank-milk *Salmonella* Dublin ELISA ODC% in dairy herds. *Prev. Vet. Med.* 68:165–179.
- Nielsen, P. K., M. B. Petersen, L. R. Nielsen, T. Halasa, and N. Toft. 2015. Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma Bovis*. *Prev. Vet. Med.* 121:338–342.
- Pinho, L., G. Thompson, M. Machado, and J. Carvalheira. 2013. Management practices associated with the bulk tank milk prevalence of *Mycoplasma* spp. in dairy herds in Northwestern Portugal. *Prev. Vet. Med.* 108:21–27.
- Pinnow, C. C., J. A. Butler, K. Sachse, H. Hotzel, L. L. Timms, and R. F. Rosenbusch. 2001. Detection of *Mycoplasma bovis* in preservative-treated field milk samples. *J. Dairy Sci.* 84:1640–1645.
- Punyapornwithaya, V., L. K. Fox, D. D. Hancock, J. M. Gay, and J. R. Aldredge. 2010. Association between an outbreak strain causing mycoplasma bovis mastitis and its asymptomatic carriage in the herd: A case study from Idaho, USA. *Prev. Vet. Med.* 93:66–70.
- Taurel, A. F., R. Guatteo, A. Joly, and F. Beaudreau. 2012. Relationship between the level of antibodies in bulk tank milk and the within-herd seroprevalence of *Coxiella burnetii* in cows. *Epidemiol. Infect.* 140:1710–1713.
- Thomas, C. B., P. Willeberg, and D. E. Jasper. 1981. Case-control study of bovine mycoplasmal mastitis in California. *Am. J. Vet. Res.* 42:511–515.