Interpretive summary

Analysis of bulk tank milk antibodies against *Mycoplasma bovis*. Petersen. *Mycoplasma bovis* is a bacterial infection associated with severe disease and production losses in cattle herds. The relevance and limitations for use of *Mycoplasma bovis* antibody measurements on bulk tank milk remain to be investigated. In this study it was found that increasing prevalence of antibody positive cows was associated with higher *Mycoplasma bovis* bulk tank milk ELISA values.The prevalence of antibody positive young stock did not correlate with the bulk tank milk ELISA values. In conclusion some, but not all, *Mycoplasma bovis* infected dairy herds are detectable by bulk tank milk ELISA-testing.

BULK TANK MILK ANTIBODIES TO MYCOPLASMA BOVIS

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

Mette B. Petersen*[1], Kaspar Krogh† and Liza R. Nielsen*

*University of Copenhagen, Faculty of Health and Medical Sciences, Department of Large Animal Sciences, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark

†Kaspar Krogh, Veterinary Cattle Specialist, Kolind, Denmark

Corresponding author: Mette Bisgaard Petersen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark, Phone +45 35 32 06 94, Fax +45 35 28 30 22, e-mail mbp@sund.ku.dk.

ABSTRACT

The relevance and limitations for using measurements of antibodies against *Mycoplasma bovis* (*M. 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 sero-prevalence is a result of on-going or recent spread of *M. bovis* in a dairy herd, we tested the hypothesis that increasing prevalences 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 four different herd level infection groups (8 control herds, 14 acute outbreak herds, 7 herd with previous outbreaks and 10 herds with elevated BTM ELISA-values directed against *M. bovis* (>64 ODC%)) were visited 4-5 times approximately 3 m 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).

A linear mixed effects model was used to analyse 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 (ODC%). In contrast, the prevalence of antibody positive young stock did not correlate with the BTM ODC%.

In conclusion, some *M. bovis* associated herd infections are detectable by BTM ELISA-testing, but there are limitations and further investigations of the effect of different clinical disease expressions in the herds are warranted.

Key words: *Mycoplasma bovis*, enzyme-limked immunosorbent assay (ELISA), bulk tank milk, antibody.

INTRODUCTION

Mycoplasma bovis (***M. bovis***) can cause severe disease and production losses in both dairy and beef producing cattle herds. In adult cattle *M. bovis* infection is often associated with mastitis, but also arthritis and pneumonia can be seen. In calves the typical disease manifestations are otitis media, pneumonia and/or arthritis (Maunsell et al., 2011). *M. 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 has not been considered a major pathogen in Danish cattle prior to 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 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 polymerase chain reaction (PCR) has become more widely used, since 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 individual level, antibodies directed against *M. bovis* can be detected in serum and milk 1-2 weeks after uptake of the bacteria (Boothby et al., 1987; Byrne et al., 2005), but the use for diagnosis in individual animals is not always straight forward (Maunsell et al., 2011). *M. 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 to 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 enzyme linked immunosorbent assay (ELISA) against PCR for BTM for national screening purposes, the use of antibodies in BTM for diagnosing either disease or presence of *M. bovis* in specific dairy herds has not been addressed in published

77 literature. Antibody measurements on BTM have been used as a diagnostic tool for the control of
78 other infectious diseases, because it can be easy and inexpensive to use in national surveillance
79 programs (Lindberg and Alenius, 1999; Nielsen, 2013). But in order to use antibodies against *M.*
80 *bovis* in BTM for surveillance purposes it is essential to know which factors influence the antibody
81 level in BTM.

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

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

98

99 **MATERIALS AND METHODS**

100 ***Populations***

101 The target population was all Danish dairy herds enrolled in the voluntary milk recording system[1],
102 which at the beginning of the study period consisted of approximately 3000 (90% of all) Danish
103 dairy herds. Their average annual milk yield per cow was 9,663 kg milk and the average herd size
104 was 166 lactating cows. The study population consisted of herds about which the Knowledge
105 Centre for Agriculture (now 'SEGES') had prior knowledge about *M. bovis* associated disease
106 either from farmers or veterinarians. SEGES is the merger of the former Knowlegde Centre for
107 Agriculture and the Danish Pig Research Centre, effective as per 1 January 2015. The company is
108 owned by the farmers and provides knowledge, consultancy and technology to all Danish farmers[2].

109 Only herds with more than 100 dairy cows were included. More than 100 cows were needed to
110 make sure the herd had enough young stock to sample. The study population consisted of 39 dairy
111 herds selected by a veterinarian at SEGES during the period March 2013-February 2014. The
112 veterinarian at SEGES had prior knowledge about the herds from national screenings in 2012 and
113 2013, where BTM from all dairy herds were tested for antibodies against *M. bovis* and with PCR, as
114 well as information provided by the local consulting veterinarian in the herds. To ensure collection
115 of data from herds with different severity and duration of disease, the following criteria were used
116 to select herds to fit into 1 of 4 groups prior to enrollment in the field data collection part of the
117 study:

118 Control herds: negative in diagnostic tests (PCR, ELISA and BC), no history of clinical signs that
119 could be related to *M. bovis* over the past 3 years, 8 herds.

120 Case herds – acute: Recent clinical suspicion of disease associated with *M. bovis,* 14 herds. In these
121 14 herds, the presence of *M. bovis* was confirmed by positive *M. bovis* PCR (PathoProof (Ct<37))

[1] https://www.landbrugsinfo.dk/Kvaeg/RYK/Sider/RYK_English.aspx
[2] http://www.seges.dk/English/AboutSEGES/AboutKCA.htm?WBCMODE=ptqqewbamqsp

samples from individual cows and/or BTM. In 4 herds it was measured in BTM, in 5 herds at individual cow level and in 5 herds at both individual cow level and BTM. In addition, 5 of the 14 herds were positive for *M. bovis* in BC of samples from individual animals.

Case herds – previous: Previous clinical suspicion of disease associated with *M. bovis.* This group included herds with former *M. bovis* test positive clinically ill animals, but that no longer had any acutely diseased animals, 7 herds. 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 value against *M. bovis* in BTM (Bio-X Bio K 302 ELISA value >64 ODC%) 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 ODC% measurements from the different herds over time, divided into the abovementioned 4 categories are 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 this 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 and/or spread of *M. bovis*.

Each herd was visited 4-5 times approximately 3 mo apart. At each visit 65 young stock equally distributed in the age group 0-12 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 Vejen, Denmark). A sample coefficient was calculated as: ODC% = (OD sample - OD negative control)/(OD positive control - OD negative control) x 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. The test has to the authors' knowledge 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 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. This variable was calculated as the proportion of cows with individual-ELISA ODC% ≥ 37 in milk out of all tested cows in the herd on the sampling d.

The apparent prevalence of antibody positive young stock. This variable was 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 d.

The apparent prevalence of antibody positive lactating cows > 50 ODC%. To assess if there was an effect 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. Herd size was calculated as the average number of cows in the herd, in the quarter of the yr 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 days 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 in order to evaluate whether there were linear relationships 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 backwards stepwise elimination of non-significant variables and their 2-way interactions. The criteria for keeping a

189 variable in the model was $p < 0.05$, and the model fit was assessed by Akaike´s Information Criteria
190 (AIC), the lower AIC the better model. The *p*-values were calculated as an ANOVA comparison
191 between a model with all variables and a model without the specific variable and its interaction
192 terms.

193 Herd was included as a random effect to account for clustering of BTM samples originating from
194 the same herd. The explanatory degree of the model was assessed by calculation of the ratio: (R_e-
195 R_{fm}/R_e), where R_e is the residual variance of the model only containing the random effect of herd
196 and R_{fm} is the residual variance of the final model.

197 Data management and analyses were made using "R: A language and environment for statistical
198 computing"[3] version 3.0.2.

199

200 **RESULTS**

201 ***Descriptive Statistics***

202 Data selection yielded 113 observations distributed on 37 herds with 2-5 observations per herd, on
203 average 3 observations per herd. Descriptive statistics of the outcome, *M. bovis* BTM ODC% and
204 explanatory variables are shown in Table 1.

205

206 A visual presentation of the raw data is provided in Figure 2, where the BTM ELISA ODC% is
207 plotted against the apparent prevalence of antibody positive lactating cows.

208

[3] (www.r-project.org)

When adding the prevalence of antibody positive young stock to the dataset, many observations were lost when limiting the prevalence calculation to +/- 30 d from the BTM date. Therefore another dataset 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 vs. observed values visually. The final model explained 54% of the variation (Table 3).

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, eventhough there may be a tendency towards overestimation of the high values, and underestimation of the low BTM values.

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 with increasing number of antibody positive cows in the herd, 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) in addition 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 consistant systematic recordings of clinical disease associated with *M. bovis* in individual animals, which would have been interesting to study the effect of.

Eventhough the prevalence of antibody positive cows is associated with the BTM ODC%, it is more ambiguous than seen with other diseases. In our dataset 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 straight forward, 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 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. There is also a lack of investigations of the correlation between antibodies in milk and serum in the literature, but the manufacturer of the used ELISA test states in a data-sheet about the test that the correlation is 0.59. In an unpublished field study from Denmark 1442 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 on-going disease in a dairy herd. A better understanding of the correlations between different clinical signs, extretion 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 this study. Other studies have found an increasing probability of isolating *M. bovis* by BC from the BTM with 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

277 in BTM ODC%, while the other studies have investigated risk factors for a BC-positive BTM. With
278 increasing herd size there is a risk that the contribution of antibodies to the BTM by 1 cow becomes
279 diluted (Nekouei et al. 2015). For *Salmonella* Dublin a better explanation of the BTM ODC% was
280 found when using the mean yield-corrected ODC%, also indicating a dilution effect in the BTM
281 (Nielsen and Ersbøll, 2005). This was not the case in our study.

282 As mentioned earlier, *M. bovis* can give rise to a variety of clinical signs in different age groups,
283 and we can discuss whether or not a BTM sample will be able to detect all types of disease
284 manifestations in a herd. Two questions arise from this: i) is it possible to detect disease among
285 young stock in the BTM, and ii) is it possible to detect all types of disease manifestations among
286 cows in the BTM. We included the prevalence of antibody positive young stock as an explanatory
287 variable to partially clarify this issue. The prevalence of antibody positive young stock did not
288 correlate with the BTM ELISA ODC%, indicating that the status of young stock is not reflected in
289 the BTM. Hence, to determine the status of the young stock, samples from individual animals are
290 probably needed. Further studies on this matter are definitely warranted.
291 The other part of this question is whether or not disease among cows manifested primarily as e.g.
292 arthritis will be detectable in a BTM sample. Unfortunately, we do not have systematically recorded
293 information about the prevalence of the different disease manifestations in the different herds, so
294 this issue cannot be further elucidated in this study. Further studies where the distinction in the
295 expression of clinical disease can be made are warranted.
296
297 Another model with the prevalence of lactating cows based on ELISA50 as the explanatory variable
298 instead of the prevalence at the recommended cut-off at 37 ODC% was tried. This did not change
299 the model fit when the other explanatory variables were the same (results not shown). The reason
300 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, and 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 with high cut-off values, because there were few cows with higher ELISA-responses.

To the best of the authors' 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 dataset. The results of that study and the present study complement each other. Our study illustrates that the lack of Se may be due to the fact that quite high prevalences of affected animals are required for the BTM antibody level to increase. As discussed above the results from our study are in overall agreement with similar studies about other infectious diseases such as *Salmonella* Dublin, bovine viral diarrhea 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 in order to use BTM-ELISA testing as a tool in herd level *M. bovis* diagnosis of dairy herds.

Data Quality and Availability

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 dataset 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 dataset, 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 month (Nicholas et al. 2002). Nontheless, 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

The 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 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.

FUNDING

The Danish Milk and Cattle Levy Funds provided funding for the sampling and laboratory testing. The funding source was not involved in the study design, analyses and interpretation, nor in the decision to publish the study.

ACKNOWLEDGEMENTS

Thank you to all the farmers participating in the *M. bovis* project. Thanks to veterinarians Ulla Torpe, Johanne Juul and Camilla Eskerod Kristensen for contribution to the *M. bovis* project including collection of samples and outbreak information from the herds. We also take the opportunity to thank two peer-reviewers for comments that helped us improve the manuscript.

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(1):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(24):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(6):537-545.

Friis, N. F. 1984. Mycoplasma-bovis-induced mastitis in cattle in Denmark. Nord. Vet. Med. 36(9-10):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(4):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.

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.

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. Article in press.

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.

394 Nielsen, P. K., M. B. Petersen, L. R. Nielsen, T. Halasa and N. Toft. 2015. Latent class analysis of
395 bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma Bovis.* Prev.
396 Vet. Med. 121(3-4):338-342.

397 Pinho, L., G. Thompson, M. Machado, and J. Carvalheira. 2013. Management practices associated
398 with the bulk tank milk prevalence of *Mycoplasma* spp. in dairy herds in Northwestern
399 Portugal. Prev. Vet. Med. 108(1):21-27.

400 Pinnow, C. C., J. A. Butler, K. Sachse, H. Hotzel, L. L. Timms, and R. F. Rosenbusch. 2001.
401 Detection of *Mycoplasma bovis* in preservative-treated field milk samples. J. Dairy Sci.
402 84(7):1640-1645.

403 Punyapornwithaya, V., L. K. Fox, D. D. Hancock, J. M. Gay, and J. R. Alldredge. 2010.
404 Association between an outbreak strain causing mycoplasma bovis mastitis and its
405 asymptomatic carriage in the herd: a case study from Idaho, USA. Prev. Vet. Med. 93(1):66-
406 70.

407 Taurel, A. F., R. Guatteo, A. Joly, and F. Beaudeau. 2012. Relationship between the level of
408 antibodies in bulk tank milk and the within-herd seroprevalence of *Coxiella burnetii* in
409 cows. Epidemiol. Infect. 140:1710-1713.

410 Thomas, C. B., P. Willeberg, and D. E. Jasper. 1981. Case-control study of bovine mycoplasmal
411 mastitis in California. Am. J. Vet. Res. 42(3):511-515.
412

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

	Min	Q1	Median	Q3	Max
BTM *M. bovis* 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

416
417

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

Clinical signs	Min	Q1	Median	Q3	Max
Prevalence of antibody positive young stock (≥37 ODC%)					
	0.00	0.12	0.28	0.38	0.66

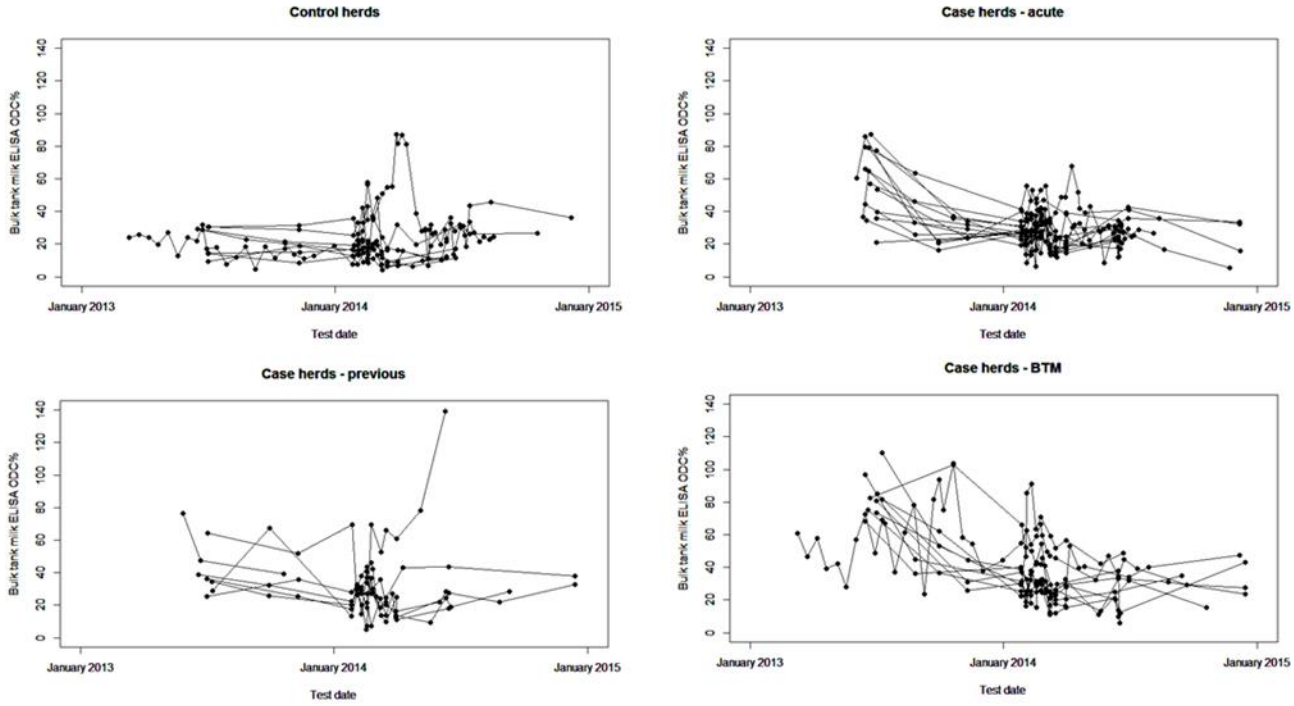
420
421

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

Variables (Explains 54% of the variation)			
Random effects	Variance		S.D.
Herd	19		4
Residuals	80		9
Fixed effects	Estimate	S.E.	P-value
BTM ELISA ODC% (intercept)	17	1.4	-
Prevalence of AB positive lactating cows (per 10% increase)	9	0.7	< 0.001

424
425

426 **Figure 1.**



427
428

429

430

431

432

433

434

435

436

437

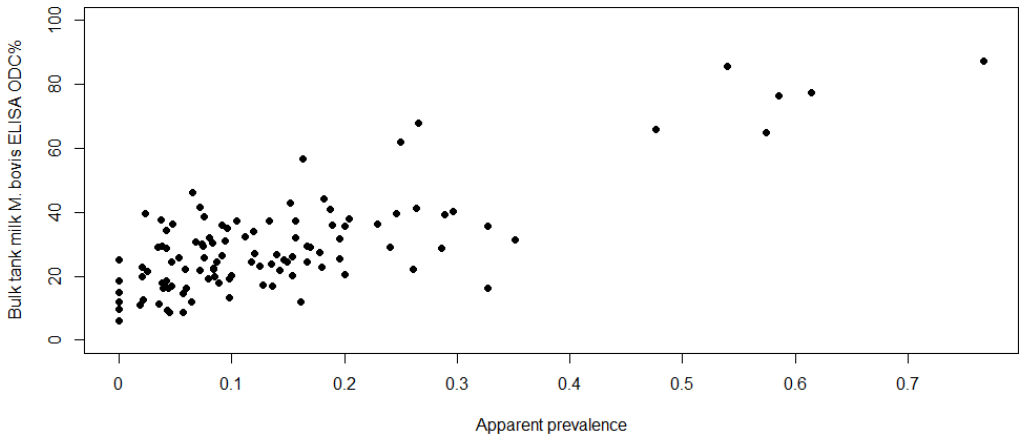
438

439

440

441 Petersen, Figure 1

442 **Figure 2.**



443

444

445

446

447

448

449

450

451

452

453

454

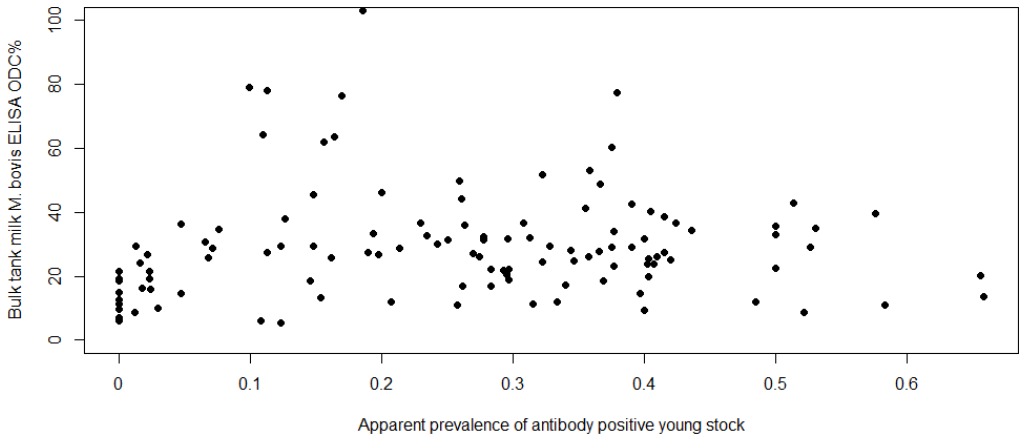
455

456

457

458 Petersen, Figure 2

459 **Figure 3.**



460

461

462

463

464

465

466

467

468

469

470

471

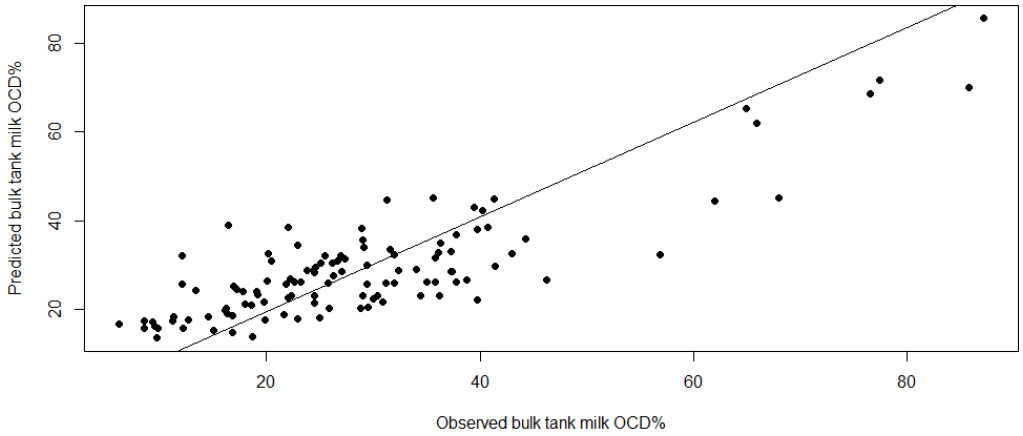
472

473

474

475 Petersen, Figure 3

476 **Figure 4.**



477

478

479

480

481

482

483

484

485

486

487

488

489

490

491

492 Petersen, Figure 4

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

497

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

501

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

504

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

508
509