

1 **Interpretive summary**

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

10

11 **BULK TANK MILK ANTIBODIES TO MYCOPLASMA BOVIS**

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

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21 **ABSTRACT**

22 The relevance and limitations for using measurements of antibodies against *Mycoplasma bovis* (*M.*
23 *bovis*) in bulk tank milk (BTM) as a potentially cost-effective diagnostic tool for herd classification
24 has not been evaluated before. Assuming that an increasing or high sero-prevalence is a result of
25 on-going or recent spread of *M. bovis* in a dairy herd, we tested the hypothesis that increasing
26 prevalences of antibody positive cows and young stock are associated with increasing BTM
27 antibody ELISA values against *M. bovis* in Danish dairy herds with different courses of *M. bovis*
28 infection. Furthermore, we tested whether herd size was associated with variations in the BTM
29 responses.

30 Thirty-nine Danish dairy herds selected to represent four different herd level infection groups (8
31 control herds, 14 acute outbreak herds, 7 herd with previous outbreaks and 10 herds with elevated
32 BTM ELISA-values directed against *M. bovis* (>64 ODC%)) were visited 4-5 times approximately
33 3 m apart. At each visit 65 young stock were blood sampled. At the milk recording date closest to
34 the herd visit date, 50 milk recording samples from individual lactating cows were randomly
35 selected. In addition a BTM sample was collected as a representative sample directly from the bulk
36 tank by the dairies' milk truck drivers as part of the mandatory milk quality control scheme. Blood
37 and milk samples were tested for antibodies against *M. bovis* with a commercially available ELISA
38 test (Bio-X BIO K 302).

39 A linear mixed effects model was used to analyse the effects of the prevalence of antibody positive
40 lactating cows and young stock and herd size on the BTM *M. bovis* ELISA results. Herd was
41 included as a random effect to account for clustering of BTM samples originating from the same
42 herd.

43 Increasing prevalence of antibody positive lactating cows was the only variable associated with
44 increasing *M. bovis* BTM ELISA optical density measurement (ODC%). In contrast, the prevalence
45 of antibody positive young stock did not correlate with the BTM ODC%.

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

49

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

52

INTRODUCTION

53

54 *Mycoplasma bovis* (***M. bovis***) can cause severe disease and production losses in both dairy and beef
55 producing cattle herds. In adult cattle *M. bovis* infection is often associated with mastitis, but also
56 arthritis and pneumonia can be seen. In calves the typical disease manifestations are otitis media,
57 pneumonia and/or arthritis (Maunsell et al., 2011). *M. bovis* seems to be an emerging pathogen in
58 countries all over the world, and even though *M. bovis* was first isolated in Denmark in 1981 (Friis,
59 1984), it has not been considered a major pathogen in Danish cattle prior to 2011. However, the
60 Danish cattle industry has had increased focus on this infection over the last couple of years due to
61 an increase in the number of severe outbreaks of *M. bovis* associated disease on herd-level.

62 Traditionally *M. bovis* has been detected by bacteriological culture (**BC**) from either individual milk
63 samples or bulk tank milk (**BTM**) samples. In recent years detection by polymerase chain reaction
64 (PCR) has become more widely used, since it is less time consuming and apparently can produce
65 similar sensitivity and specificity to conventional BC methods (Pinnow et al., 2001; Cai et al.,
66 2005). At individual level, antibodies directed against *M. bovis* can be detected in serum and milk
67 1-2 weeks after uptake of the bacteria (Boothby et al., 1987; Byrne et al., 2005), but the use for
68 diagnosis in individual animals is not always straight forward (Maunsell et al., 2011). *M. bovis* can
69 also be isolated from asymptomatic carrier animals (Punyapornwithaya et al., 2010), but it is not
70 known how the antibody response in these animals reacts compared to clinically ill animals.

71 However, in beef cattle, group-level antibody titers and seroconversion can be associated with
72 active infection (Martin et al., 1990), and spread of the disease in a dairy herd could therefore be
73 expected to lead to a marked increase in seroprevalence. Except for Nielsen et al. (2015), who
74 evaluated the performance of an antibody detecting enzyme linked immunosorbent assay (ELISA)
75 against PCR for BTM for national screening purposes, the use of antibodies in BTM for diagnosing
76 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 system¹,
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 Knowledge 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 farmers².

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/RVK/Sider/RVK_English.aspx

² <http://www.seges.dk/English/AboutSEGES/AboutKCA.htm?WBCMODE=ptqgewbamqsp>

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

125 Case herds – previous: Previous clinical suspicion of disease associated with *M. bovis*. This group
126 included herds with former *M. bovis* test positive clinically ill animals, but that no longer had any
127 acutely diseased animals, 7 herds. In these 7 herds, the presence of *M. bovis* was confirmed by
128 positive PCR (PathoProof PCR (Ct<37)) in milk samples from individual cows and BTM in 3
129 herds, and in BTM in 4 herds.

130 Case herds – BTM: High ELISA value against *M. bovis* in BTM (Bio-X Bio K 302 ELISA value
131 >64 ODC%) in a national screening in summer 2013, 10 herds.

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

136 The distribution of BTM ODC% measurements from the different herds over time, divided into the
137 abovementioned 4 categories are shown in Figure 1. We aimed to include herds of different sizes
138 and geographical locations. However, systematic stratification according to these factors was not
139 used. More than 90 % of the Danish dairy cattle are located on the peninsula of Jutland, and all
140 herds enrolled in this study were located in Jutland. Because the prevalence of *M. bovis* infection is
141 low, the selection criteria were used to ensure inclusion of herds with evidence of disease and/or
142 spread of *M. bovis*.

143 Each herd was visited 4-5 times approximately 3 mo apart. At each visit 65 young stock equally
144 distributed in the age group 0-12 mo old were blood sampled. At the milk recording date closest to

145 the herd visit date, 50 milk recording samples from individual lactating cows were randomly
146 selected. A BTM was sampled as a representative sample while the bulk tank was emptied by the
147 dairies' milk truck drivers as part of the mandatory milk quality control scheme.

148

149 ***Detection of Antibodies***

150 Milk samples from both individual animals and BTM, and serum samples from the young stock
151 were analyzed for antibodies against *M. bovis* using the commercial kit Bio-X BIO K 302
152 *Mycoplasma bovis* ELISA kit at Eurofins-Steins Laboratory (Holstebro and Vejen, Denmark). A
153 sample coefficient was calculated as: $ODC\% = (OD \text{ sample} - OD \text{ negative control}) / (OD \text{ positive}$
154 $\text{control} - OD \text{ negative control}) \times 100 \%$, where OD is the optical density measured by the ELISA
155 reader for each test sample, and negative and positive control samples on the sample ELISA plate.
156 For animal-level testing a sample coefficient ≥ 37 ODC% was considered positive, and a sample
157 coefficient < 37 ODC% was considered negative according to the recommendations of the
158 manufacturer of the ELISA kit. The test has to the authors' knowledge not been evaluated with
159 regard to sensitivity (Se) and specificity (Sp) for animal level diagnosis in the field.

160 It has been evaluated for use on BTM in national screening of dairy herds for national or regional
161 prevalence estimation by Nielsen et al. (2015). The Se and Sp at cut-off 37 ODC% were 60.4 and
162 97.3, respectively. At a cut-off of 50 ODC% the Se 43.5 and the Sp was 99.6.

163 ***Description of Variables***

164 The outcome variable was the continuous *M. bovis* BTM ODC%.

165 Four explanatory variables were tested as potential explanatory variables of the *M. bovis* BTM
166 ODC%.

167 ***The apparent prevalence of antibody positive lactating cows.*** This variable was calculated as
168 the proportion of cows with individual-ELISA ODC% ≥ 37 in milk out of all tested cows in the
169 herd on the sampling d.

170 ***The apparent prevalence of antibody positive young stock.*** This variable was calculated as
171 the proportion of young stock with individual-ELISA ODC% ≥ 37 in blood out of all tested young
172 stock in the herd on the sampling d.

173 ***The apparent prevalence of antibody positive lactating cows > 50 ODC%.*** To assess if there
174 was an effect of the ELISA cut-off used for apparent prevalence calculations, the apparent
175 prevalence was also calculated as the proportion of cows with individual-ELISA ODC% > 50
176 (ELISA50) in milk.

177 ***Herd Size.*** Herd size was calculated as the average number of cows in the herd, in the quarter
178 of the yr where the BTM sample was collected.

179

180 An observation was excluded if it was not possible to match the date of the apparent prevalence
181 with a BTM sample within +/- 30 days or if the number of animals for the prevalence calculations
182 was low ($N < 30$).

183 ***Statistical Analysis***

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

187 Two linear mixed effects models were created. The models were built by backwards stepwise
188 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)

209 When adding the prevalence of antibody positive young stock to the dataset, many observations
210 were lost when limiting the prevalence calculation to +/- 30 d from the BTM date. Therefore
211 another dataset was created that only contained the prevalence of antibody positive young stock and
212 the BTM samples closest to the date of the prevalence calculation (n=116). Descriptive statistics of
213 the young stock prevalence are shown in Table 2. From Figure 3 it is apparent that the prevalence of
214 antibody positive young stock did not correlate well with the BTM *M. bovis* ELISA ODC%, and the
215 variable was therefore not included in further analysis.

216

217 *Analytical Statistics*

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

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

224

225 The predicted *M. bovis* ELISA ODC% in BTM is plotted against the observed values in Figure 4.
226 Overall the model predicted the BTM values well, eventhough there may be a tendency towards
227 overestimation of the high values, and underestimation of the low BTM values.

228

229

DISCUSSION

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

233 The prevalence of antibody positive lactating cows was positively associated with the BTM ODC%.
234 Each time the prevalence increased by 10% the BTM ODC% increased by 9 ODC%. This means
235 that with increasing number of antibody positive cows in the herd, indicative of recent spread of *M.*
236 *bovis* bacteria, we can expect the BTM ODC% to increase. This association is in agreement with
237 other studies on other infectious diseases in dairy herds (Nielsen and Ersbøll, 2005; Muskens et al.,
238 2011; Taurel et al., 2012). For *Salmonella* Dublin, Nielsen and Ersbøll (2005) in addition found that
239 the degree of explanation increased when including the prevalence or number of high ELISA-
240 responders and whether or not the herd had had a positive BC for *Salmonella* Dublin. In our study,
241 the prevalence of high ELISA-responders could not be included in the same model as the
242 prevalence and unfortunately we did not have sufficient BC-results for *M. bovis* from all farms or
243 comprehensive and consistent systematic recordings of clinical disease associated with *M. bovis* in
244 individual animals, which would have been interesting to study the effect of.

245 Eventhough the prevalence of antibody positive cows is associated with the BTM ODC%, it is more
246 ambiguous than seen with other diseases. In our dataset and according to our final model, the
247 prevalence of antibody positive cows was above 30% before the BTM on average went above the
248 cut-off of 37 ODC% (Table 3 and Figure 2) indicating that a large proportion of the cows had to
249 have been exposed to *M. bovis* to make the BTM antibody testing able to detect it with reasonably
250 Se and Sp (Nielsen et al., 2015). This hampers the ability to classify herds based on a BTM sample.
251 A more persistent pattern has been found for *Coxiella burnetii* measurements in BTM where all
252 samples above the cut-off value had a within-herd prevalence of at least 20% (Muskens et al.,
253 2011). The discrepancy may arise because many *M. bovis* clinically diseased and medically treated

254 cows do not contribute to the bulk tank. The apparent prevalence in our study stems from samples
255 from individual cows at milk recording. Most of these cows would have contributed to the BTM on
256 the day they were sampled. A minor part of medically treated cows could also have been part of
257 milk recording, but the milk from those cows would not have entered the BTM due to procedures
258 for preventing antibiotic residues entering the milk for consumption.

259 As mentioned in the introduction, the use of antibodies to detect disease among individual animals
260 is not straight forward, and clinical disease is not always followed by a rise in antibodies (Maunsell
261 et al., 2011). Unfortunately, evaluation of antibody reactions in individual animals in field studies is
262 sparse. On group level, however, antibody titers show correlation with disease in beef cattle (Martin
263 et al., 1990), which would suggest that the same could be the case for dairy herds. There is also a
264 lack of investigations of the correlation between antibodies in milk and serum in the literature, but
265 the manufacturer of the used ELISA test states in a data-sheet about the test that the correlation is
266 0.59. In an unpublished field study from Denmark 1442 paired serum and milk samples from 8
267 dairy herds had a correlation of 0.7. When considering the different clinical manifestations of *M.*
268 *bovis* disease, it could be that antibodies in milk are not a good measure of on-going disease in a
269 dairy herd. A better understanding of the correlations between different clinical signs, excretion of
270 bacteria and serum and milk antibodies would help interpret the BTM antibody response.

271

272 Herd size was not associated with the BTM ODC% in herds in this study. Other studies have found
273 an increasing probability of isolating *M. bovis* by BC from the BTM with increased number of
274 lactating cows (Thomas et al., 1981; Pinho et al., 2013). This is probably related to the different
275 outcomes in the studies, and the fact that in our model the presence of *M. bovis* is already taken into
276 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

301 ELISA cut-off at animal-level with regard to detection of infected or infectious animals within
302 infected herds. A higher cut-off might detect more truly infected animals as opposed to previously
303 exposed animals, and hence the ELISA50-prevalence might be better correlated with the BTM-
304 antibody level. However, this did not seem to be the case. We did not try with high cut-off values,
305 because there were few cows with higher ELISA-responses.

306

307 To the best of the authors' knowledge no studies have evaluated antibodies in BTM as a diagnostic
308 tool for *M. bovis* in relation to the underlying disease manifestation in dairy herds. Nielsen et al.
309 (2015) evaluated the overall performance of the BTM-test method for national or regional screening
310 purposes and provided estimates of Se, Sp and predictive values. However, the estimates were
311 associated with much uncertainty due to few test-positive herds in the dataset. The results of that
312 study and the present study complement each other. Our study illustrates that the lack of Se may be
313 due to the fact that quite high prevalences of affected animals are required for the BTM antibody
314 level to increase. As discussed above the results from our study are in overall agreement with
315 similar studies about other infectious diseases such as *Salmonella* Dublin, bovine viral diarrhoea
316 virus and *Coxiella burnetii* infections (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et
317 al., 2012). However, we also found some challenges that have to be addressed in order to use
318 BTM-ELISA testing as a tool in herd level *M. bovis* diagnosis of dairy herds.

319

320 **Data Quality and Availability**

321 In most instances, the prevalence estimates were not based on the same date of sampling, but within
322 +/- 30 d of the BTM sample. Hence, we cannot be certain that milk from all the individual cows
323 used for calculating the prevalence was present in the BTM sample. To evaluate the limitation of

324 this, a dataset consisting of 87 of the observations (75%) sampled within +/- 14 d of the BTM
325 sample were used to rerun the final model. This rerun model yielded approximately the same
326 estimates as the model based on the larger dataset, and did not make the predictions for the model
327 better. Hence, our final model appeared to be robust to the uncertainties in the prevalence
328 estimation related to the time of BTM sampling. In individual animals the antibody response can
329 persist for at least 6 month (Nicholas et al. 2002). Nonetheless, from our data it seems to be
330 important to realize that the BTM antibody level is actually quite dynamic, and a high response in
331 BTM does not necessarily persist for long time (Figure 1).

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

338

339

CONCLUSIONS

340 The objective was to identify factors that influence the variation in BTM ELISA ODC% against *M.*
341 *bovis* in Danish dairy herds. Increasing prevalence of antibody positive cows was associated with
342 increasing *M. bovis* BTM ELISA ODC%. In contrast, the prevalence of antibody positive young
343 stock did not correlate with the BTM ODC%. Herd size was not associated with *M. bovis* BTM
344 ELISA ODC%. A combination with distinction between different clinical signs would be very
345 interesting, but the available data did not support such investigation. More studies to investigate risk
346 factors for variance in BTM ELISA ODC% for *M. bovis* and potential combinations of test-

347 procedures to use for herd classifications are warranted before this method can be deemed useful for
348 disease control purposes.

349

350

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354

355

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360

REFERENCES

361

362 Boothby, J. T., D. E. Jasper, and C. B. Thomas. 1987. Experimental intramammary inoculation with
363 *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on local and systemic
364 antibody response. *Can. J. Vet. Res.* 51(1):121-125.

365 Byrne, W., B. Markey, R. McCormack, J. Egan, H. Ball, and K. Sachse. 2005. Persistence of
366 *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated
367 experimentally. *Vet. Rec.* 156(24):767-771.

368 Cai, H. Y., P. Bell-Rogers, L. Parker, and J. F. Prescott. 2005. Development of a real-time PCR for
369 detection of *Mycoplasma bovis* in bovine milk and lung samples. *J. Vet. Diagn. Invest*
370 17(6):537-545.

371 Friis, N. F. 1984. *Mycoplasma-bovis*-induced mastitis in cattle in Denmark. *Nord. Vet. Med.* 36(9-
372 10):324-325.

373 Lindberg, A. L. E., and S. Alenius. 1999. Principles for eradication of bovine viral diarrhoea virus
374 (BVDV) infections in cattle populations. *Vet. Microbiol.* 64:197-222.

375 Martin, S.W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac and M. Thorburn. 1990. A
376 Group Level Analysis of the Associations Between Antibodies to Seven Putative Pathogens
377 and Respiratory Disease and Weight Gain in Ontario Feedlot Calves. *Can J Vet Res.*
378 54:337-342.

379 Maunsell, F. P., A. R. Woolums, D. Francoz, R. F. Rosenbusch, D. L. Step, D. J. Wilson and E. D.
380 Janzen. 2011. *Mycoplasma bovis* infections in cattle. *J. Vet. Intern. Med.* 25(4):772-783.

381 Muskens, J., E. v. Engelen, C. v. Maanen, C. Bartels, and T. J. G. M. Lam. 2011. Prevalence of
382 *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and
383 individual samples by PCR and ELISA. *Vet. Rec.* 168:79.

384 Nicholas, R. A. J., R. D. Ayling and L. P. Stipkovits. 2002. An experimental vaccine for calf
385 pneumonia by *Mycoplasma bovis*: clinical, culture, serological and pathological findings.
386 *Vaccine.* 20:3569-3575.

387 Nekouei, O., H. Stryhn, J. VanLeeuwen, D. Kelton, P. Hanna and G. Keefe. 2015. Predicting
388 within-herd prevalence of infection with bovine leukemia virus using bulk-tank milk
389 antibody levels. *Prev. Vet. Med.* Article in press.

390 Nielsen, L. R. 2013. Review of pathogenesis and diagnostic methods of immediate relevance for
391 epidemiology and control of *Salmonella* Dublin in cattle. *Vet. Microbiol.* 162:1-9.

392 Nielsen, L. R., and A. K. Ersbøll. 2005. Factors associated with variation in bulk-tank-milk
393 *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 <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

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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

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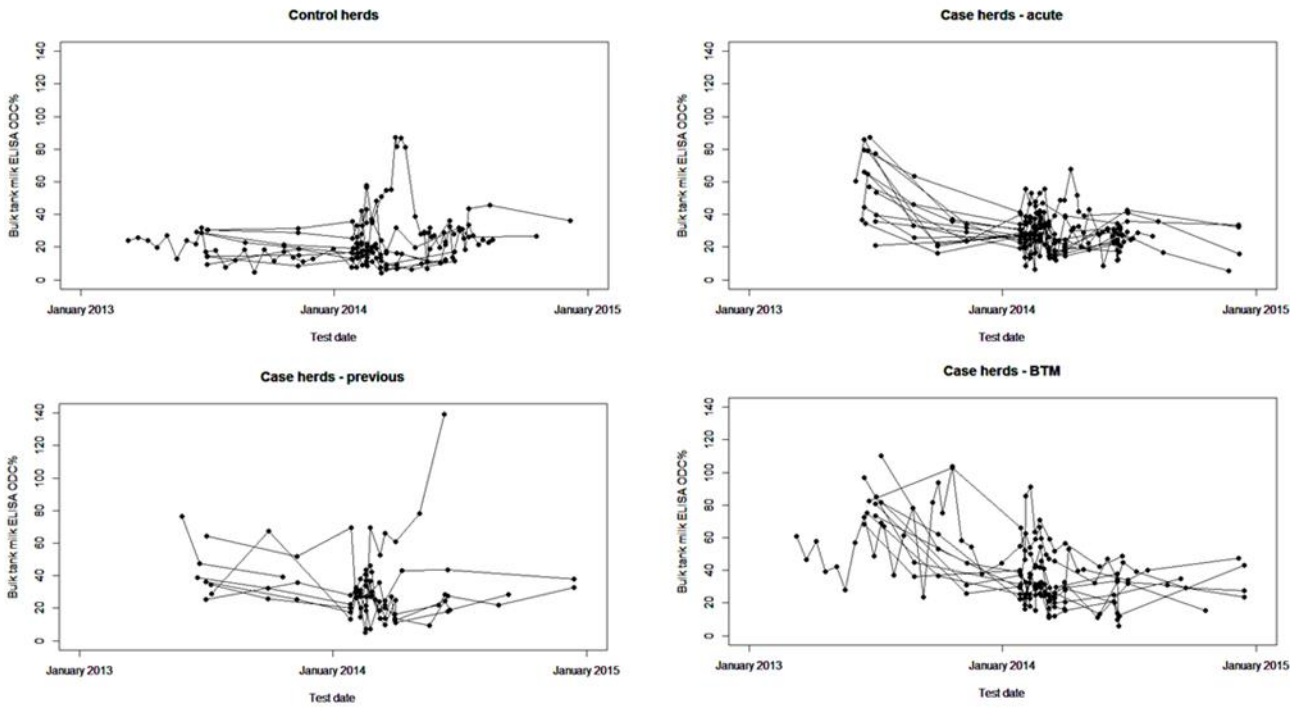
422 **Table 3.** Results of the final model describing explanatory variables and random effects of bulk
 423 tank milk (BTM) ELISA optical density measurement (ODC%) for *Mycoplasma bovis*

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

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426 **Figure 1.**



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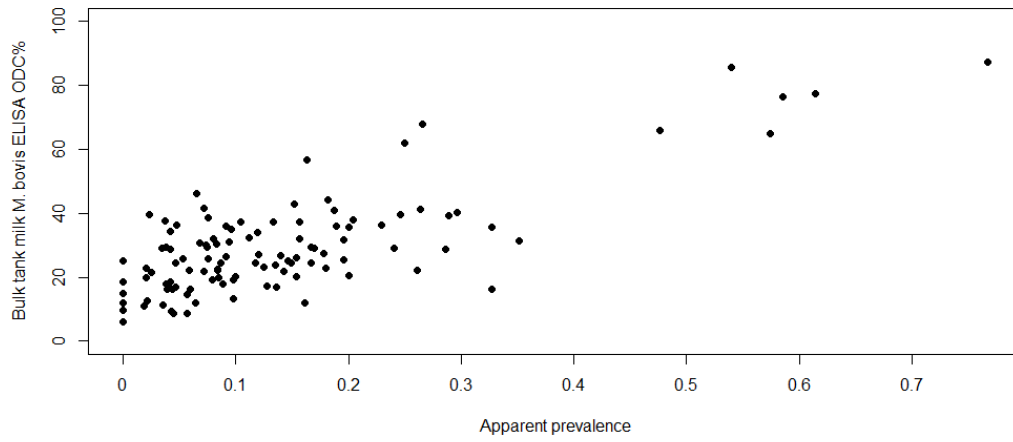
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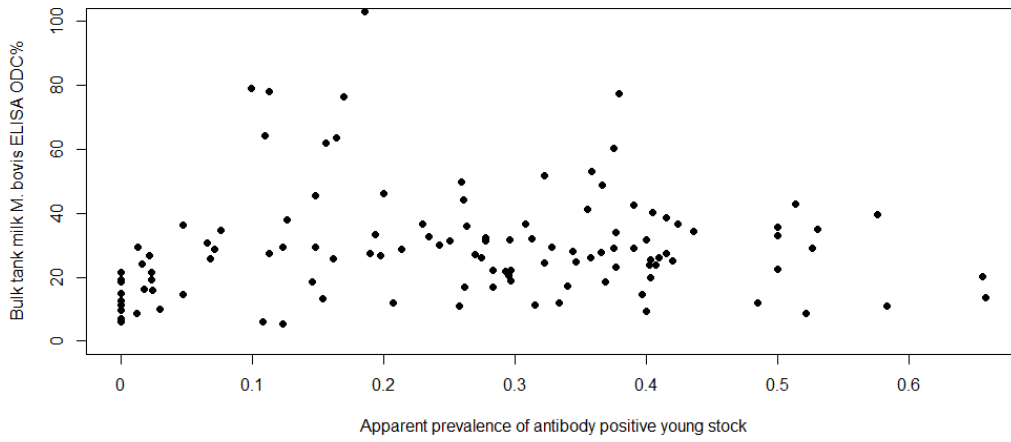
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Petersen, Figure 1

442 **Figure 2.**



459 **Figure 3.**



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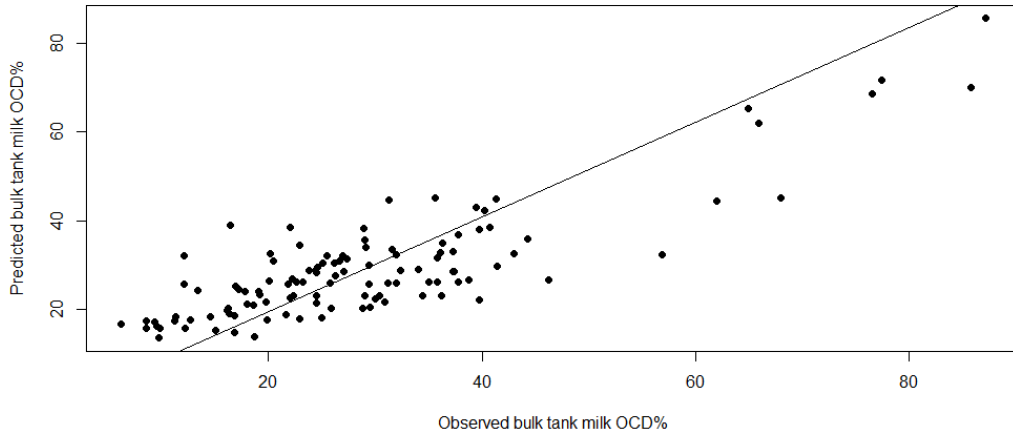
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Petersen, Figure 3

476 **Figure 4.**



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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.

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509