Preventive Veterinary Medicine 121 (2015) 338–342

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*

Per Kantsø Nielsen[a,*], Mette Bisgaard Petersen[b], Liza Rosenbaum Nielsen[b], Tariq Halasa[a], Nils Toft[a]

[a] Technical University of Denmark, National Veterinary Institute, Section for Epidemiology, Bülowsvej 27, 1870 Frederiksberg C, Denmark
[b] University of Copenhagen, Faculty of Health and Medical Sciences, Department of Large Animal Sciences, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 7 April 2015
Received in revised form 26 June 2015
Accepted 14 August 2015

Keywords:
Suggestions
Mycoplasma bovis
ELISA
PCR
Latent class analysis
Sensitivity
Specificity

ABSTRACT

The bacterium *Mycoplasma bovis* causes disease in cattle of all ages. An apparent increase in the occurrence of *M. bovis* associated outbreaks among Danish dairy cattle herds since 2011 has prompted a need for knowledge regarding herd-level diagnostic performance. Therefore, the objective of this study was to evaluate the herd-level diagnostic performance of an indirect ELISA test by comparison to a real-time PCR test when diagnosing *M. bovis* in cattle herds of bulk tank milk.

Bulk tank milk samples from Danish dairy herds ($N = 3437$) were analysed with both the antibody detecting BIO K 302 *M. bovis* ELISA kit and the antigen detecting PathoProof Mastitis Major-3 kit. As none of these are considered a gold standard test for herd-level diagnostics we applied a series of Bayesian latent class analyses for a range of ELISA cut-off values. The negative and positive predictive values were calculated for hypothetical true national prevalences (1, 5, 10, 15 and 20%) of infected herds.

We estimated that the ELISA test had a median sensitivity and specificity of 60.4 [37.5–96.2 95% Posterior Credibility Interval] and 97.3 [94.0–99.8 95% PCI] at the currently recommended cut-off (37% Optical density Coefficient). These changed to 43.5 [21.1–92.5 95% PCI] and 99.6 [98.8–100 95% PCI] if the cut-off was increased to 50 ODC%. In addition, herd-level diagnosis by ELISA would result in fewer false positives at a cut-off value of 50 ODC% compared to 37 ODC% without compromising the negative predictive value.

1. Introduction

The bacterium *Mycoplasma bovis* (*M. bovis*) causes disease in cattle of all ages. In young calves, *M. bovis* is commonly associated with otitis media, pneumonia and arthritis. In cows, it is primarily reported in cases with mastitis (Maunsell et al., 2011; Nicholas and Ayling, 2003). *M. bovis* can cause chronic infections that are difficult to treat and it can also be carried asymptomatically (Brown et al., 1990; Byrne et al., 2005 Fox et al., 2005). The movement of carrier animals allows *M. bovis* to spread unnoticed between farms and this inevitably lead to production losses and impairment of animal welfare. A US estimate on the annual costs of *M. bovis* infection was made in 1999, where it was found to exceed US$ 130 million for the US dairy and beef industry (Rosengarten and Citti, 1999). In Europe, the *M. bovis* herd-level prevalence has previously been estimated to be between 1.5% and 5.4% based on bacteriological culture of bulk tank milk (BTM) (Filioussis et al., 2007; Passchyn et al., 2012; Pinho et al., 2013). The last published study on Danish herd-level prevalence found 10 out of 227 (4.4%) BTM samples from different herds positive by culture (Friis, 1984) and the current herd-level prevalence is unknown. Recently a number of Danish dairy farms have reported severe outbreaks of *M. bovis*, and this has resulted in a renewed focus on the bacterium within the Danish farming community. If a potential future surveillance or control programme is to be initiated an evaluation of the applied diagnostic test methods must be performed. This has not been done for herd-level diagnostics of *M. bovis* with the current diagnostic methods.

Herd-level diagnosis of *M. bovis*, based on bacteriological culture, has been applied to BTM samples in both routine surveillance- and eradication efforts (Passchyn et al., 2012; Pinho et al., 2013). However, this method can be relatively expensive compared to more modern methods. More cost-effective alternative diagnostic methods based on either polymerase chain reaction (PCR) or enzyme-linked immunosorbent assays (ELISA) are commercially available. Some PCR based tests have been applied for herd-level diagnosis of *M. bovis* in BTM samples (Arcangioli et al., 2011; Justice-

* Corresponding author. Fax: +45 35886001.
E-mail address: pkani@vet.dtu.dk (P.K. Nielsen).

http://dx.doi.org/10.1016/j.prevetmed.2015.08.009
0167-5877/

Allen et al., 2011) and have shown similar sensitivity (Se) and specificity (Sp) to bacterial culture (Cai et al., 2005). A number of ELISAs for animal-level diagnostics of M. bovis are described in the literature (Ghadersohi et al., 2005 Uhaa et al., 1990). None of these have, to the best of the authors' knowledge, been evaluated for use on BTM samples.

The diagnostic performance of a test is commonly evaluated against a reference test, which is assumed to be a gold standard with perfect Se and Sp. A latent class analysis (LCA) allows the estimation of Se and Sp of the evaluated diagnostic tests, in a population where the underlying true infection status is unknown (Hui and Walter, 1980). The infection dynamics of *M. bovis* within a herd make it likely that the analytes of an antibody detecting ELISA and a PCR test will be present simultaneously in a BTM sample (Pfützner and Sachse, 1996). The presence of these two analytes could thereby reflect an underlying infection state of the herd, whenever an *M. bovis* outbreak is ongoing.

Therefore the objective of this study was to estimate the performance of an ELISA test (BIO K 302 *M. bovis* ELISA kit, Bio-X Diagnostics, Jemelle, Belgium) by comparison to a PCR test (PathoProof Mastitis Major-3 kit, Thermo Fisher Scientific, Helsinki, Finland) for herd-level diagnostics of *M. bovis*, using LCA. The effect on the Se and Sp, by applying different cut-off values for the ELISA when keeping the PCR cut-off constant, was evaluated and the positive and negative predictive values (PPV and NPV) were calculated at a range of hypothetical true prevalences of *M. bovis* infected dairy herds.

2. Materials and methods

2.1. Sample material

BTM samples from all Danish dairy herds were collected during the period from August 27 to November 28, 2013. The BTM samples were collected by the milk hauler, using standardized procedures, to ensure representative sampling of the whole bulk tank of milk. The farmers were not informed of when the samples would be collected. This procedure is a part of the Danish milk quality control scheme and is routinely performed during milk collection for the dairy plant. All samples were tested with both the ELISA and the PCR assay in a blinded setup, at the Eurofins Steins A/S Laboratory, Holstebro, Denmark as described in the following sections.

In order for a sample to be included in the analysis, it had to have been collected during the study period and successfully analysed with both tests on the same sample. All farms with paired test results had their geographical location as UTM (EUREF89, Zone N 32) *X* and *Y* coordinates included in the dataset. The data were divided into a north and south sub-population based on a line at the UTM coordinates 6300000 north, as required by the analysis assumptions (see Latent Class Analysis section). This split is required to obtain the minimal degrees of freedom in the data. The division was based on industry reports of a high proportion of *M. bovis* cases in Northern Jutland, compared to the rest of Denmark.

All data were obtained from the Knowledge Center for Agriculture, Cattle, Aarhus, Denmark.

2.2. PCR test

The qPCR test, Thermo Scientific PathoProof Mastitis-3 kit, Thermo Fisher Scientific, Helsinki, Finland, was performed according to the manufacturer's instructions described in the kit manual. Thus, the cycle threshold (Ct) cut-off value was set to 37, meaning that Ct values <37 were considered positive and Ct values ≥37 were considered negative.

2.3. ELISA test

The ELISA, Bio-X BIO K 302 *M. bovis* ELISA kit, Bio-X Diagnostics, Jemelle, Belgium, was performed according to the manufactures instructions described in the *M. bovis* ELISA Kit insert. In brief, the kit is an indirect ELISA with recombinant protein of *M. bovis* coated wells. Positive and negative control samples were included on the plates for quality control and calculation of the sample coefficient. The coefficient is calculated by: ODC% = (OD sample – OD negative control)/(OD positive control – OD negative control) × 100%. When applied to animal level samples, a sample coefficient ≥37 ODC% is considered positive, and a sample coefficient <37 ODC% is considered negative by the manufacturer.

2.4. Latent class analysis

In order to evaluate the two diagnostic tests, we performed a series of Bayesian latent class analyses (Branscum et al., 2005) based on the two populations, two tests conditional independence model (Hui and Walter, 1980). The model assumptions were (1) the data consisted of two subpopulations with different true prevalence, (2) the Se and Sp of the two tests were constant across the subpopulations, and (3) the tests were conditionally independent given the true disease state. Each LCA was performed for a range of ELISA cut-off values: 20, 30, 37, 40, 50 and 60 ODC%, where the 37 ODC% is the manufacturers recommended cut-off for animal-level testing. The PCR cut-off was kept constant at sample positive <37Ct, for all estimates. The Se and Sp were compared between tests by calculating the Bayesian posterior probabilities (POPR) of the two hypotheses H_0: $Se_{ELISA} \geq Se_{PCR}$ and H_0: $Sp_{ELISA} \geq Sp_{PCR}$. This was evaluated as the proportion of Markov Chain Monte Carlo (MCMC) samples where the hypotheses were true. Based on the Se and Sp, as well as a range of simulated true herd-level prevalences (1%, 5%, 10%, 15% and 20%), we calculated the PPV and NPV for the ELISA.

The posterior distributions for all parameters were sampled for 20,000 iterations, after the initial 10,000 burn-in iterations were discarded. We applied uninformative beta prior distributions, i.e. with both shape parameters set to one for the Se and Sp of both tests and for the true prevalence parameter in each of the two sub-populations. In order to diagnose the MCMC sampling, three randomly seeded Markov chains were generated for each analysis. MCMC sampling diagnostics was performed with time-series- and autocorrelation-plots of the respective chains, and by Gelman–Rubin diagnostics as suggested by (Toft et al., 2007). The analysis was carried out in OpenBUGS version 3.2.2 rev 1063 (Lunn et al., 2000) and R version 3.0.2 (R Core Team, 2013).

3. Results

Of the 3,523 BTM samples collected in the study period, 86 samples were excluded due to lack of sample material or because they were resamplings from an already sampled farm. This left 3,437 samples from Danish dairy cattle farms for the analysis (i.e. more than 95% of the active dairy farms in Denmark at the sampling time).

Fig. 1 and Table 1, respectively, show the distribution of paired test outcomes and their counts when divided into the two subpopulations for increasing ELISA cut-offs. The apparent herd-level prevalence in the two subpopulations was 9.7% (north) and 6.7% (south) for the ELISA, and 2.0% (north) and 1.5% (south) for the PCR. The nation-wide apparent prevalence was 7.2% and 1.6% by ELISA and PCR, respectively, at the manufacturers recommended cut-offs for animal-level diagnosis.

The effects of increasing the ELISA cut-off can be seen in Table 2. As expected, increasing the ELISA cut-off value resulted in lower Se

Table 1
Observed counts of paired test outcomes (ELISA/PCR: +/+, +/−, −/+, −/−) per sub-population.

		ELISA / PCR							
		North				South			
		+/+	+/−	−/+	−/−	+/+	+/−	−/+	−/−
ELISA	20	10	331	0	162	42	1592	6	1294
Cut-	30	7	93	3	400	31	426	17	2460
off	37	4	44	6	448	21	176	27	2710
value	40	4	27	6	466	19	119	29	2766
	50	2	11	8	482	13	27	35	2859
	60	2	3	8	490	8	15	40	2871

Table 2
Sensitivity (Se), specificity (Sp) and true sub-population prevalence (Tp) median estimates with 95% Posterior Credibility Intervals (PCI).

	Test								Sub-population			
	ELISA				PCR				North		South	
Parameter	Se		Sp		Se		Sp		Tp		Tp	
ELISA cut-off	Median [95% PCI]											
20	94.7	[82.9;99.8]	65.1	[49.9;96.5]	3.8	[2.2;8.5]	99.7	[99.3;100]	56.0	[33.9;75.5]	35.6	[13.1;59.4]
30	79.7	[59.4;98.7]	91.5	[85.5;99.4]	12.0	[5.9;45.2]	99.6	[99.2;100]	15.8	[4.2;28.8]	10.0	[2.4;21.1]
37	60.4	[37.5;96.2]	97.3	[94;99.8]	15.3	[8.2;48.4]	99.5	[98.9;100]	11.4	[3.2;23.2]	6.7	[1.8;14.5]
40	58.1	[34.5;96.1]	97.6	[95.6;99.8]	24.5	[11.4;82]	99.5	[98.8;100]	6.4	[1.4;15.3]	4.1	[1;10.4]
50	43.5	[21.1;92.5]	99.6	[98.8;100]	36.9	[20.2;84.5]	99.3	[98.7;100]	4.5	[1.1;10.8]	2.2	[0.7;5.4]
60	33.2	[12.7;90.4]	99.7	[99.3;100]	53.3	[24.8;95.6]	99.2	[98.5;100]	2.3	[0.5;6.7]	1.4	[0.4;4.4]

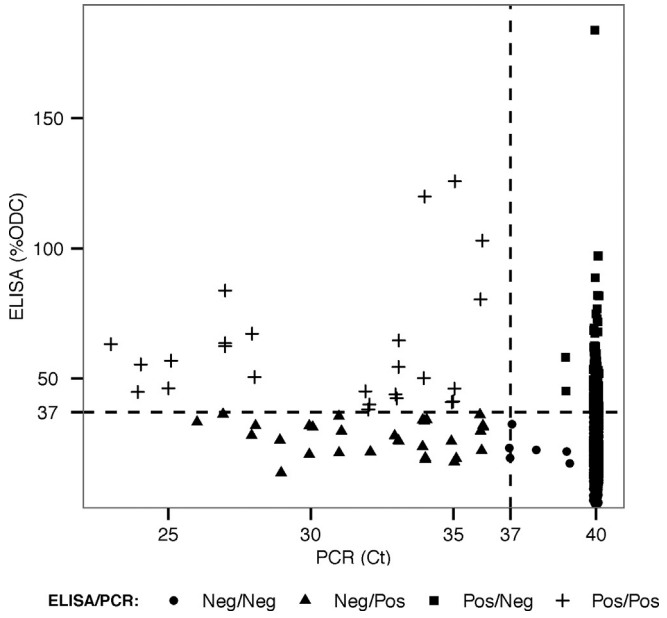

Fig. 1. Test results for 3437 bulk milk tank samples. The dotted lines indicate the manufacturers recommended cut-offs for the respective test at animal level. Cut-off values for herd-level diagnosis are not available. Data points have been 'jittered' to facilitate visualisation.

and higher Sp estimates for the ELISA. However, for PCR the Se was observed to increase and the Sp to decrease when increasing the ELISA cut-off value. For the $Se_{ELISA} \geq Se_{PCR}$ hypothesis, the POPR, value was higher than 0.95 for all cut-offs lower than 40 ODC%. That is, the ELISA Se had a probability of more than 95% of being higher than the PCR Se, at those cut-off values. The POPR was 0.90, 0.63 and 0.23 at cut-off values of 40, 50 and 60 ODC%. None of the POPR-values for the $Sp_{ELISA} \geq Sp_{PCR}$ hypothesis were above 0.95. However the probabilities diverged markedly between the 40 and 50 ODC% cut-offs. At cut-offs lower than or equal to 40 ODC%, the POPR was at most 0.08 and it changed to at least 0.67 at 50 ODC% or above. The true prevalence in both sub-populations was observed to decrease with a higher ELISA cut-off.

Fig. 2 shows the ELISA predictive values a cut-off of 37 and 50 ODC%. The major difference in the predictive test performance between the two cut-offs was in the estimated PPVs. The median PPV estimates were consistently higher at the high cut-off value of 50 ODC%. This would translate into the following estimates of false negatives and false positives in a hypothetical population of 3,437 dairy farms, where the true herd-level prevalence is 5%: The median estimates of false negative farm diagnoses would increase from 3.7 farms (0.4–5.6; 95% PCI) to 5.0 (0.6–6.9; 95% PCI) if the cut-off was increased from 37 to 50 ODC%. The median number of false positive herd diagnoses would decrease from 78.9 farms (8.3–118.7; 95% PCI) to 23.5 (1.3–68.9; 95% PCI).

4. Discussion

4.1. Result summary

We have evaluated the herd–level performance of the Bio-X BIO K 302 *M. bovis* ELISA kit, which is currently in use for screening of Danish dairy farms on BTM samples, by comparison to the Thermo Scientific PathoProof Mastitis-3 PCR kit. The evaluation was done using a Bayesian LCA, thereby omitting the need to consider either test as a perfect reference test.

The analysis found that the ELISA had higher median Se, except at 60 ODC%. The ELISA median Sp was more comparable to the PCR test Sp, when its cut-off value was increased above the manufacturer's recommended cut-off value for animal-level diagnostics. The effect of increasing the cut-off appears to have limited impact on the ELISA NPV, but the PPV appears to improve markedly by changing the cut-off from 37 to 50 ODC%.

A change in cut-off value will affect the underlying disease definition in the LCA and a comparison of the test performance across the evaluated cut-offs should therefore be done with caution. However, the POPR values do show that a cut-off of 50 ODC% is the lowest value where the ELISA is more probable to have a higher Se than

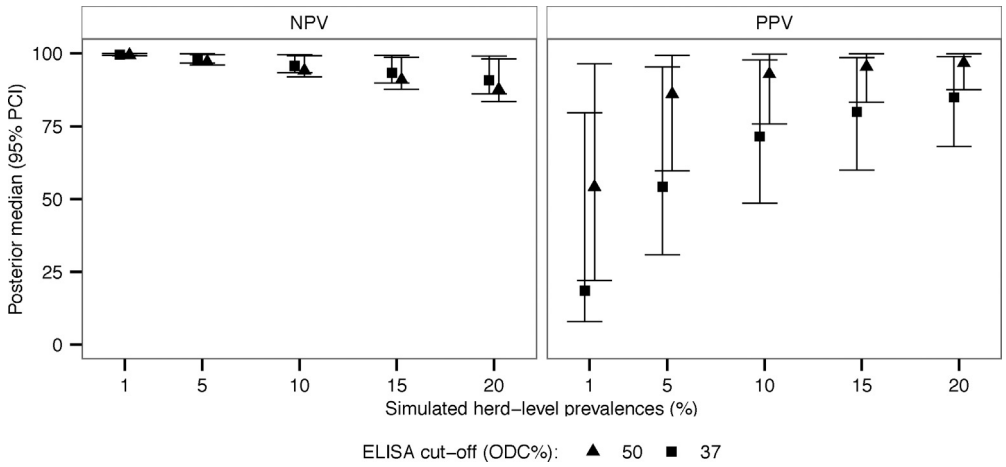

Fig. 2. Median estimates for the positive (PPV) and negative (NPV) predictive values of the ELISA test (95% PCI) at two different cut-off values (37 and 50 ODC%).

the PCR. This is furthermore the lowest ELISA cut-off where the Sp is probabilistically in favour of the ELISA.

4.2. Latent herd-level infection state

The fundamental prerequisite for evaluating diagnostic tests by LCA is the measurement of the same latent infection state by the tests under evaluation. In this case, when establishing a herd-level diagnosis of *M. bovis* from BTM samples, it is necessary to discuss the latent state, as the PCR test is limited to detection of free *M. bovis* DNA in a milk sample whereas the ELISA has the capability to detect antibodies directed against *M. bovis* secreted in the milk. This implies that the latent herd-level infection state in this study is defined by a situation with simultaneous presence of both target analytes in a BTM sample. These two events are not guaranteed to reflect the same latent state at animal-level, e.g. it is not known whether arthritis leads to *M. bovis* DNA in the milk. However, a BTM sample can be representative for the herd-level *M. bovis* infection status because it represents a combination of different animal-level infections. It is expected, that all infected animals will go through at least one phase where either bacterial DNA, antibodies, or a combination, is excreted through their milk (Pfützner and Sachse, 1996). At herd-level it therefore becomes more likely that a situation which reflects the latent herd-level infection state occurs, as more animals within a herd become infected and thereby a larger combination of animal-level infection types becomes present.

At animal-level IgA and IgG concentrations remain elevated at least 57 days post experimental inoculation (Bennett and Jasper, 1980) and an animal's antibody levels would remain higher after seroconversion compared to prior to infection. This might carry over into a higher antibody signal at herd-level and thereby lead to false positive ELISAs relative to the latent definition. This would explain the high number of samples with high ODC% values but a Ct value of 40 (Fig. 1). Likewise, a short period could exist prior to an antibody response, where a PCR result would be false positive relative to the infection state. We assumed that this period was short enough not to affect the results. However, false positive reactions may occur in the PCR test due to carry-over of DNA-material from other infected farms in the sampling equipment or at the automated handling of the samples at the laboratory. This may be part of the explanation for the slightly lower Sp of the PCR compared to the ELISA.

It is apparent that the latent disease definition is dependent on the chosen cut-off values. Significant linear correlations between BTM ELISA measurements and the within-herd prevalence have been found for other diseases. However, other factors can also influence the BTM ELISA values and make the interpretation and use of ELISA tests on herd-level difficult (Nielsen and Ersbøll, 2005; Nielsen and Toft, 2014). None of these studies investigated *M. bovis*, but were conducted on milk samples from Danish dairy herds and with indirect ELISAs. The same trend is also seen for *M. bovis* (Unpublished results). The test parameters at a chosen cut-off value should therefore be interpreted as representing a latent infection level that reflects a certain level of infected animals in the herd, but the variation can also be influenced by other factors.

4.3. Assumptions

The general effects of violating the LCA assumptions has previously been examined thoroughly in the literature (Toft et al., 2005). Using LCA for diagnostic test evaluation implies that the same latent herd-level infection status is measured by both tests and that the tests are conditionally independent given disease status (CID). The evaluated tests aim to detect infection with *M. bovis* by different biological mechanisms, thus supporting the CID assumption.

The true herd-level prevalence (Table 2) decreased for increasing ELISA cut-off values and intuitively these estimates should be constant. The change in prevalence is a consequence of the change in the definition of the underlying true infection status when the ELISA cut-off is altered. The effect caused by changing the latent infection definition, has been observed in other LCA test evaluations on BTM samples (Mweu et al., 2012). In our case the change in infection definition should be interpreted as observing a herd with a higher level of infection at increasing ODC% values, because a higher proportion of infected cows would lead to a higher BTM antibody concentration. Therefore, the prevalence estimates are dependent on the level of infection in the herds. Despite the changing prevalence estimates, differences between the north and south populations were apparent for all cut-offs. The effect of this is also responsible for the change in PCR Se and Sp estimates at the different ELISA cut-off levels.

If the homogeneity of the Danish dairy cattle population and the non-biological type of split are taken into consideration, then the assumption of having constant Se and Sp across the two subpopulations is likely to be valid.

5. Conclusion

We have evaluated the Bio-X BIO K 302 *M. bovis* ELISA kit, which is currently in use for screening of Danish dairy farms, using BTM samples against the Thermo Scientific PathoProof Mastitis-3

PCR kit. We estimated that the ELISA would have more favourable Sp and keep having a better Se, compared to the PCR, if the cut-off was increased to at least 50 ODC%, rather than using the manufacturer's recommended value suggested for animal-level diagnosis (37 ODC%). The NPV is comparable for both cut-off values, but the number of false positives would be reduced at a higher cut-off, thereby improving the PPV.

Acknowledgements

We would like to acknowledge the contribution made by the Knowledge Center for Agriculture, Cattle, in supplying the data, and to veterinarians Kaspar Krogh and Jørgen Katholm for assistance and scientific discussions about the practical implications of using the two evaluated tests in BTM screenings of Danish dairy cattle farms.

The Danish Levy Fund provided the 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.

References

Arcangioli, M.A., Chazel, M., Sellal, E., Botrel, M.A., Bezille, P., Poumarat, F., Calavas, D., Le Grand, D., 2011. Prevalence of *Mycoplasma bovis* udder infection in dairy cattle: preliminary field investigation in southeast France. N. Z. Vet. J. 59, 75–78, http://dx.doi.org/10.1080/00480169.2011.552856.

Bennett, R.H., Jasper, D.E., 1980. Bovine mycoplasmal mastitis from intramammary inoculations of small numbers of *Mycoplasma bovis*: local and systemic antibody response. Am. J. Vet. Res. 41, 889–892.

Branscum, A.J., Gardner, I.A., Johnson, W.O., 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. Prev. Vet. Med. 68, 145–163, http://dx.doi.org/10.1016/j.prevetmed.2004.12.005.

Brown, M.B., Shearer, J.K., Elvinger, F., 1990. Mycoplasmal mastitis in a dairy herd. J. Am. Vet. Med. Assoc. 196, 1097–1101.

Byrne, W., Markey, B., McCormack, R., Egan, J., Ball, H., Sachse, K., 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. Vet. Rec. 156, 767–771.

Cai, H.Y., Bell-Rogers, P., Parker, L., Prescott, J.F., 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, http://dx.doi.org/10.1177/104063870501700603.

Filioussis, G., Christodoulopoulos, G., Thatcher, A., Petridou, V., Bourtzi-Chatzopoulou, E., 2007. Isolation of Mycoplasma bovis from bovine clinical mastitis cases in Northern Greece. Vet. J. 173, 215–218, http://dx.doi.org/10.1016/j.tvjl.2005.08.001.

Fox, L.K., Kirk, J.H., Britten, A., 2005. Mycoplasma mastitis: a review of transmission and control. J. Vet. Med. B. Infect. Dis. Vet. Public Health 52, 153–160, http://dx.doi.org/10.1111/j.1439-0450.2005.00845.x.

Friis, N.F., 1984. *Mycoplasma-bovis*-induced mastitis in cattle in Denmark. Nord. Vet. Med 36, 324–325.

Ghadersohi, A., Fayazi, Z., Hirst, R.G., 2005. Development of a monoclonal blocking ELISA for the detection of antibody to *Mycoplasma bovis* in dairy cattle and comparison to detection by PCR. Vet. Immunol. Immunopathol. 104, 183–193, http://dx.doi.org/10.1016/j.vetimm.2004.11.008.

Hui, S.L., Walter, S.D., 1980. Estimating the error rates of diagnostic tests. Biometrics 36, 167–171.

Justice-Allen, A., Trujillo, J., Goodell, G., Wilson, D., 2011. Detection of multiple Mycoplasma species in bulk tank milk samples using real-time PCR and conventional culture and comparison of test sensitivities. J. Dairy Sci. 94, 3411–3419, http://dx.doi.org/10.3168/jds.2010-3940.

Lunn, D.J., Thomas, A., Best, N., Spiegelhalter, D., 2000. WinBUGS—A Bayesian modelling framework: Concepts, structure, and extensibility. *Statistics and Computing* 10, 325–337.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D., 2011. *Mycoplasma bovis* infections in cattle. J. Vet. Intern. Med. 25, 772–783, http://dx.doi.org/10.1111/j.1939-1676.2011.0750.x.

Mweu, M.M., Toft, N., Katholm, J., Nielsen, S.S., 2012. Evaluation of two herd-level diagnostic tests for Streptococcus agalactiae using a latent class approach. Vet. Microbiol. 159, 181–186, http://dx.doi.org/10.1016/j.vetmic.2012.03.037.

Nicholas, R.A.J., Ayling, R.D., 2003. *Mycoplasma bovis*: disease, diagnosis, and control. Res. Vet. Sci. 74, 105–112.

Nielsen, L.R., Ersbøll, K.A., 2005. Factors associated with variation in bulk-tank-milk Salmonella Dublin ELISA ODC% in dairy herds. Prev. Vet. Med. 68, 165–179, http://dx.doi.org/10.1016/j.prevetmed.2004.12.006.

Nielsen, S.S., Toft, N., 2014. Bulk tank milk ELISA for detection of antibodies to *Mycobacterium avium* subsp. paratuberculosis: correlation between repeated tests and within-herd antibody-prevalence. Prev. Vet. Med. 113, 96–102, http://dx.doi.org/10.1016/j.prevetmed.2013.10.013.

Passchyn, P., Piepers, S., De Meulemeester, L., Boyen, F., Haesebrouck, F., De Vliegher, S., 2012. Between-herd prevalence of *Mycoplasma bovis* in bulk milk in Flanders, Belgium. Res. Vet. Sci. 92, 219–220, http://dx.doi.org/10.1016/j.rvsc.2011.03.016.

Pfützner, H., Sachse, K., 1996. *Mycoplasma bovis* as an agent of mastitis pneumonia, arthritis and genital disorders in cattle. Rev. Sci. Technol. 15, 1477–1494.

Pinho, L., Thompson, G., Machado, M., Carvalheira, J., 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, http://dx.doi.org/10.1016/j.prevetmed.2012.07.001.

R Core Team, 2013. R: A language and environment for statistical computing.

Rosengarten, R., Citti, C., 1999. The role of ruminant mycoplasmas in systemic infection. Mycoplasmas ruminants Pathog. diagnostics. Epidemiol. Mol. Genet. 3, 14–17.

Toft, N., Innocent, G.T., Gettinby, G., Reid, S.W.J., 2007. Assessing the convergence of Markov Chain Monte Carlo methods: an example from evaluation of diagnostic tests in absence of a gold standard. Prev. Vet. Med. 79, 244–256, http://dx.doi.org/10.1016/j.prevetmed.2007.01.003.

Toft, N., Jørgensen, E., Højsgaard, S., 2005. Diagnosing diagnostic tests: evaluating the assumptions underlying the estimation of sensitivity and specificity in the absence of a gold standard. Prev. Vet. Med. 68, 19–33, http://dx.doi.org/10.1016/j.prevetmed.2005.01.006.

Uhaa, I.J., Riemann, H.P., Thurmond, M.C., Franti, C.E., 1990. The use of the enzyme-linked immunosorbent assay (ELISA) in serological diagnosis of *Mycoplasma bovis* in dairy cattle. Vet. Res. Commun. 14, 279–285.