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

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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.

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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-

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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

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