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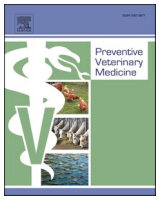

Bayesian diagnostic test evaluation and true prevalence estimation of mycoplasma bovis in dairy herds

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ABSTRACT

The true prevalence of dairy cattle herds with *M. bovis* infections in the Netherlands is unknown. Previous attempts to estimate prevalences were hampered by the absence of a diagnostic serological test that was validated under field conditions. This study estimated sensitivity and specificity of two commercial serum ELISAs and the true *M. bovis* herd prevalence using different Bayesian latent class models. A total of 7305 serum samples from 415 randomly chosen dairy herds were collected in fall/winter 2019 and investigated for presence of antibodies against *M. bovis* using the BIO-K-260 ELISA from Bio-X. Serum samples from 100 of these herds were also tested with a second ELISA, from IDvet. A Bayesian latent class model using the paired test results estimated a sensitivity of 14.1% (95% Bayesian probability interval (BPI): 11.6–16.7%) for the Bio-X ELISA and a specificity of 97.2% (95% BPI: 95.9–98.4%). Sensitivity and specificity for the IDvet ELISA were estimated at 92.5% (95% BPI: 88.3–96.5%) and 99.3% (95% BPI: 98.7–99.8%), respectively. Also, Bio-X ELISA sensitivity was considerably higher with data from calves only and with data from a selection of herds with a clinical outbreak, whereas the IDvet ELISA sensitivity was fairly constant under these conditions. These differences in test sensitivity is expected to be related to an effect of time since infection. A second Bayesian model, applied on test results of all 415 herds, estimated a true herd prevalence of 69.9% (95% BPI: 62.7–77.6%), suggesting *M. bovis* in endemic amongst dairy cattle herds in the Netherlands. To what extent seropositive herds have experienced a clinical outbreak needs further investigation.

1. Introduction

Mycoplasma bovis (*M. bovis*) has become increasingly important as a pathogen on beef and dairy cattle farms, causing welfare and production losses (Maunsell et al., 2011; Dudek et al., 2020). Since the first reported case of mastitis in 1961 (Hale et al., 1962), *M. bovis* has been detected worldwide, in all major cattle rearing countries (Dudek et al., 2020). *M. bovis* is a primary cause of mastitis, arthritis, keratoconjunctivitis and otitis media as well as a part of the bovine respiratory disease complex (BRD) (Maunsell et al., 2011). As antimicrobial treatment of *M. bovis* mastitis and arthritis is mostly unsuccessful, it is often advised to cull cattle with *M. bovis* mastitis and/or arthritis. However, *M. bovis* infections may persist in a dairy herd, also through asymptomatic carriers (Punyapornwithaya et al., 2010).

M. bovis can be identified in individual cattle milk or bulk milk samples by bacterial culture or PCR (Parker et al., 2018), although the bacterium may be missed due to variations in affected tissues between cattle, intermittent shedding in milk and withholding of milk from mastitis cows from bulk milk. Antibodies against *M. bovis* can be detected in serum and (bulk) milk using an ELISA (Parker et al., 2018). The first commercially available ELISA is produced by Bio-X (Bio-X Diagnostics S.A., Rochefort, Belgium). A second one, from IDvet (IDvet, Grabels, France), became available in 2018 and was shown to be more sensitive than the monowell K-302 ELISA from Bio-X (Andersson et al., 2019; Petersen et al., 2020; Bokma et al., 2022). At Royal GD, the double well K-260 ELISA of Bio-X with both antigen-coated wells and negative control antigen-coated wells was used for routine diagnostics at the time of this study.

Based on different diagnostic methods, herd level and animal level prevalences have been determined in different European countries and estimates vary considerably. In Belgium, true dairy herd prevalence was 31.8% as determined by a combination of PCR and ELISA in bulk milk (Gille et al., 2018). In Denmark, dairy herd prevalences were 1.6% (PCR) and 7.2% (ELISA) (Nielsen et al., 2015). In Sweden, dairy herd prevalence was 0% (PCR) and 4.8% (ELISA) (Hurri et al., 2022). Herd

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prevalence amongst Irish dairy herds is estimated to be 45% as determined by ELISA in bulk milk (McAloon et al., 2022). In the Netherlands, an increased number of *M. bovis* outbreaks have been observed in dairy cattle herds in the last decade, particularly *M. bovis*-induced cases of mastitis and arthritis. However, the true prevalence of *M. bovis* infections in the dairy cattle population is unknown. Previous attempts to estimate seroprevalences were hampered by the absence of a diagnostic test that was validated under field conditions due to lack of a gold standard. Bayesian latent class models are known for their ability to deal with this issue, in which an animal's observed status is linked to the unobserved true infection status (Johnson et al., 2019). The objectives of this study were therefore to use Bayesian modelling 1) to estimate the sensitivities and specificities of two commercially available serum ELISAs in the absence of a gold standard and 2) to estimate the prevalence of *M. bovis* infections in Dutch dairy herds.

2. Materials and methods

2.1. Study design

The study was conducted in two stages. In the first stage, a random selection of dairy herds was sampled to estimate the prevalence of *M. bovis* infection based on presence of antibodies against *M. bovis* in serum. In a second stage, serum samples from a subset of the herds were used to estimate test validity of two serum ELISA kits.

In the first stage, a sample size of 284 herds was chosen to estimate herd prevalence of *M. bovis,* based on an expected seroprevalence of 25% (Gille et al., 2018), 95% confidence and 5% error. A total of 1183 dairy farms were invited to participate, based on an expected response rate of 40% and, subsequently, 40% compliance with the required sampling strategy. Earlier work showed that with measuring antibodies in serum of a sample of about 12 lactating cows and a least 6 calves (1–6 months old) an acute *M. bovis* infection could be detected at herd level (Penterman et al., 2022). Therefore, only herds with at least 6 calves present were eligible for selection. The sample of 1183 farms were randomly chosen out of a population of 10,329 dairy herds with at least six calves 1–6 months old in July-August 2019. Farms were invited by e-mail in August 2019 to participate in the survey in fall/winter 2019–2020. Participating farmers were requested to arrange with their private practitioner to collect blood samples from 12 randomly selected cattle of at least 2 years old and six calves of 1–6 months old at sampling. Blood samples were to be collected between October 1 and December 31, 2019. Samples were investigated for presence of *M. bovis*-specific antibodies using the BIO-K-260 ELISA kit from Bio-X ('ELISA A') which was routinely used at Royal GD. Test outcomes were first expressed as a sample to positive percentage (S/P%). For this, a net optical density (OD) was calculated by subtracting the OD value in the control well (with negative control antigen) from the OD of the well with *M. bovis* antigen. Additionally, according to the manufacturer, test outcomes were categorized into one of six classes:

S/P% $\leq$ 37%: 0.
37 $<$ S/P% $\leq$ 60%: + .
60 $<$ S/P% $\leq$ 83%: + +.
83 $<$ S/P% $\leq$ 106%: + ++ .
106 $<$ S/P% $\leq$ 129%: + ++ +.
S/P% $>$ 129%: + ++ ++ .

In the second stage of the study, a subset of 100 herds out of the herds from the first stage of the study were selected for a ELISA test evaluation study. Herds were selected based on an alternative test result, the IDvet ELISA on bulk milk samples which ranged from negative to high positive in bulk milk (*results not shown*). All serum samples from the 100 herds were investigated for presence of *M. bovis*-specific antibodies using the ID Screen® ELISA kit from IDvet ('ELISA B'). Test outcomes were first expressed as an S/P% as $((OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control})) \times 100\%$. According to the manufacturer, test outcomes were categorized as follows:

S/P% $<$ 60%: 0.
60 $\leq$ S/P% $<$ 80%: + .
80 $\leq$ S/P% $<$ 110%: + +.
110 $\leq$ S/P% $<$ 140%: + ++ .
S/P% $\geq$ 140%: + ++ +.

2.2. Analysis

Two Bayesian models were designed. In the first model, combined test results of the two ELISAs on serum samples from the subset of 100 farms were compared to estimate sensitivity and specificity of the ELISAs ('Model 1'). In a second model, the herd-level prevalence was estimated using test results of all herds ('Model 2'). In both models, test outcomes were first dichotomized as 'positive' (categories $>$ 0) or 'negative' (category 0) prior to analyses.

2.2.1. Model 1: test characteristics

A 2 $\times$ 2 table with the frequencies of the observed combinations of test results (A^+B^+, A^+B^-, A^-B^+, A^-B^-) made the data frame for the model. A latent class model was used for two tests in multiple populations, introduced by Hui and Walter (1980). Considering the hierarchy in the subset of 100 herds, with a fixed number of 18 paired test results per herd, we developed a model for 100 subpopulations. In the model, each herd was considered a distinct population under the assumption of equal misclassification rates across populations, yet with different prevalences in terms of seropositivity. These assumptions are fundamental in the Hui-Walter model to achieve model identifiability (Toft et al., 2005; Johnson et al., 2009). The Hui-Walter model also assumes conditional independence of test results given the disease status. In our study however, conditional dependence of ELISA A and ELISA B test outcomes was expected because both tests detect serum antibodies (Gardner et al., 2000; Georgiadis et al., 2003). The model therefore needed to be extended taking into account dependence of test results, to prevent poor inferences (Toft et al., 2005; Johnson et al., 2009). Consequently, the 2 $\times$ 2 combination of test results are stratified by the (latent) disease status D of the subjects in each subpopulation, leading to the cross-classification summarized in Table 1 (Dendukuri, 1998). Note that from the frequencies in Table 1, only the 2 $\times$ 2 subtotals N_{AB} are observed. As the latent status in this model is an immune response, 'seropositive' might be a more appropriate terminology than 'diseased'. Yet for simplicity, seropositive animals and herds are termed 'diseased' and 'infected' throughout this paper.

The conditional dependence between the two tests was estimated using the covariance between the two tests among the diseased (p) and non-diseased (n) subjects, in accordance with the model described by Dendukuri and Joseph (2001):

$$covp = cov(A, B \mid D^+) = p(A^+B^+ \mid D^+) - (SeA \times SeB) \quad (1)$$

$$covn = cov(A, B \mid D^-) = p(A^- B^- \mid D^-) - (SpA \times SpB) \quad (2)$$

According to the model, the eight multinomial cell probabilities of the cross-tabulated data were estimated as follows:

2.2.2. Infected animals

$p(A^+B^+ \mid D^+) = \pi \times (SeA \times SeB + covp)$.
$p(A^+B^- \mid D^+) = \pi \times (SeA \times (1\text{-}SeB) - covp)$.
$p(A^-B^- \mid D^+) = \pi \times ((1\text{-}SeA) \times (1\text{-}SeB) + covp)$.
$p(A^-B^+ \mid D^+) = \pi \times ((1\text{-}SeA) \times SeB - covp)$.

2.2.3. Non-infected animals

$p(A^-B^- \mid D^-) = (1\text{-}\pi) \times (SpA \times SpB + covn)$.
$p(A^-B^+ \mid D^-) = (1\text{-}\pi) \times (SpA \times (1\text{-}SpB) - covn)$.
$p(A^+B^- \mid D^-) = (1\text{-}\pi)\ ((1\text{-}SpA) \times SpB - covn)$.

$$p(A^+B^+ \mid D^-) = (1\text{-}\pi) \times ((1\text{-}SpA) \times (1\text{-}SpB) + covn) \quad (3)$$

Table 1
Conceptual cross-classification of observed (N) and latent (Y) data from two diagnostic tests in animals that are truly diseased (D+) or non-diseased (D-).

	D^+		D^-	
	ELISA A = +	ELISA A = -	ELISA A = +	ELISA A = -
ELISA B = +	Y_{++}	Y_{-+}	N_{++} - Y_{++}	N_{-+} - Y_{-+}
ELISA B = -	Y_{+-}	Y_{--}	N_{+-} - Y_{+-}	N_{--} - Y_{--}

Where π being the animal-level true prevalence of *M. bovis*, SeA, SeB and SpA, SpB are the sensitivities and specificities of ELISA A and ELISA B respectively, *covp* the covariance between the tests in diseased subjects and *covn* the covariance between the tests in non-diseased subjects (Dendukuri and Joseph, 2001).

The model was applied in four scenarios. First, the model ran on all sera from the 100 herds (Scenario 1; default). The model code for Scenario 1 can be found in supplementary file S1. Then, the model was run on test results from cows only (Scenario 2) and calves only (Scenario 3). Finally, a completely distinct selection of 128 cow sera from 5 herds with an acute clinical outbreak of *M. bovis* were used in a model with a Two Tests Five Populations design (Scenario 4). This scenario was applied to investigate the hypothesis that test sensitivity might decrease with prolonged time since infection, or increase when applied in high prevalence populations (Johnson et al., 2009). The five herds were known to have an acute clinical outbreak of *M. bovis* and were as such part of an observational study to gain insight in the within-herd dynamics of *M. bovis* (Penterman et al., 2022).

Informative prior distributions for sensitivity and specificity of ELISA A and ELISA B were obtained from literature (Table 2). These priors were chosen as they involve the same tests, are based on data that are independent of the current data, but somehow similar to it (although based on serum samples from calves only). The covariance parameters were constrained as such that the combined sensitivities and specificities of the two tests cannot exceed the individual values of the test characteristics. Non-informative priors were used for the animal-level seroprevalence in each population (herd). The 'PriorGen' package in R 4.2.1 (Kostoulas, 2018) was used to obtain shape parameters for the informative prior (beta) distributions. To assess sensitivity of the model to priors, the analysis of the default model (Scenario 1) was rerun with uninformative and weakly informative priors for SeA, SeB, SpA, and SpB. Uninformative priors were set at beta(1,1). The original informative priors were made weakly informative by giving them a wider distributional spread, suggesting a lack of knowledge (Johnson et al., 2019; Depaoli et al., 2020) (Table 2). As the original priors for the specificities were centred around 99%, the median of the weakly informative priors were lowered to 82% to enable a more diffuse distribution.

Table 2
Prior information for parameters to estimate test sensitivity and specificity of ELISA A and ELISA B, with median prior probabilities and 95% Bayesian probability interval (BPI), reference and distribution.

Parameter	Prior median (95% BPI)	Distribution	Reference (where relevant)
SeA	28% (15.3–43.7%)	beta(13, 32)	Schibrowski et al. (2018)
SeB	94% (90.0–97.0%)	beta(161.11, 10.28)	Andersson et al. (2019)
SpA	98.6% (92.9–100%)	beta(50, 1)	Schibrowski et al. (2018)
SpB	99% (98.0–99.7%)	beta(529.9, 5.35)	Andersson et al. (2019)
covp	-	uniform(lb_p, ub_p)[a]	Dendukuri and Joseph (2001)
covn	-	uniform(lb_n, ub_n)[a]	Dendukuri and Joseph (2001)
π	-	beta(1, 1)	-
Sensitivity analysis: Uninformative			
SeA	-	beta(1,1)	-
SeB	-	beta(1,1)	-
SpA	-	beta(1,1)	-
SpB	-	beta(1,1)	-
Sensitivity analysis: Weakly informative			
SeA	28% (10.0–53.1%)	beta(4.45, 10.92)	-
SeB	94% (60.0–100%)	beta(5.83, 0.65)	-
SpA	82% (50.0–97.7%)	beta(7.13, 1.78)	-
SpB	82% (50.0–97.7%)	beta(7.13, 1.78)	-

[a] lb_p is the lower bound of *covp*: (SeA-1)× (1-SeB); lb_p is the upper bound of *covp*: min(SeA, SeB)- SeA×SeB; lb_n is the lower bound of *covn*: (SpA-1)× (1-SpB); lb_n is the upper bound of *covn*: min(SpA, SpB)-SpA×SpB

From the cell probabilities in (Eq. 3) the multinomial likelihood was constructed, combined with priors, and Bayes' theorem was applied to obtain posterior distributions of the parameters. Posterior inferences were obtained with the package 'runjags' in R 4.2.1 (Denwood et al., 2016; Plummer et al., 2019; R Core Team, 2018) using Markov chains, with 10,000 iterations after a burn-in period of 5000 iterations. Convergence of the Markov chains was assessed by visual assessment of Markov chains and trace plots and by running multiple (n = 2) chains from distinct starting values (e.g., 0.05 and 0.95 for variables bounded between 0 and 1). The Brooks-Gelman-Rubin diagnostic was used to assure that the two chains had converged (Brooks and Gelman, 1998), inspecting the potential scale reduction factor being very close to 1. Effective sample sizes were monitored (>1000) to ensure that autocorrelation was not problematic.

Throughout this manuscript, Model 1 is described according to the Standards for the Reporting of Diagnostic accuracy studies that use Bayesian Latent Class Models (STARD-BLCM) (Kostoulas et al., 2017). A completed checklist can be found in supplementary file S2.

2.2.4. Model 2: herd prevalence

A Bayesian latent-class model was developed to estimate herd prevalence of *M. bovis* amongst dairy herds in the Netherlands, estimating the number of animals testing positive in each herd is a function of the within-herd animal-level true prevalence, and the test characteristics of the test used. The number of serum samples per herd, the apparent number of positive test results per herd and the ELISA kit (ELISA A or ELISA B), made the data frame for the model. Note that all herds were tested with ELISA A and a subset of 100 herds also with ELISA B. For the latter group, only the results of B were retained for the analysis. The dataset also contained the four regions of the Netherlands in which the herds were located (north, east, south and west), the farming system (open or closed), and their herd size based on the number of lactating cows. The model was inspired by a study by McAloon et al. (2016). In their study, serological test results from a national control program were used in a Bayesian latent-class model to estimate herd-level true prevalence of paratuberculosis in Ireland, taking the test characteristics of various ELISA kits used into account. The model was constructed as:

$NT_k^+ \mid p(T^+)_k, n_k \sim binomial(p(T^+)_k, n_k)$ = number of animals in herd k that tested positive, with n_k being the number of cows tested per herd (4)

$p(T^+)_k = Se_i \times \pi_k + (1\text{-}Sp_i)(1\text{-}\pi_k)$ = probability of a positive test result for an animal in herd k, based on π and the sensitivity and specificity of ELISA i (5)

$\pi_k = HTP_k \times CWHP_k$ = animal-level true prevalence ($p(D^+)$) for an animal in herd k (6)

$HTP_k \sim bernoulli(\mu)$ = herd-level infection status with μ being the probability of a herd being infected (7)

$CWHP_k \sim beta(a_{CWHP}, b_{CWHP})$ = within-herd prevalence in herd k, with shape parameters a and b (8)

$\mu \sim uniform(0,1)$ = probability of a randomly chosen herd containing on or more truly infected animals (9)

(*continued on next page*)

(*continued*)

$Se_i \sim beta(a_{Se}, b_{Se})$	= sensitivity of ELISA *i*	(10)
$Sp_i \sim beta(a_{Sp}, b_{Sp})$	= specificity of ELISA *i*	(11)

where NT_k^+ equals the number of animals testing positive in herd *k*. The probability of a randomly chosen animal from a herd testing positive ($p(T^+)_k$) was a function of the animal-level true prevalence π within herd *k*, and the diagnostic test characteristics; Se and Sp, which varied according to ELISA kit used (*i*). Shape parameters for the informative prior (beta) distributions of Se_i and Sp_i were as described in Table 2. The animal-level true prevalence π for a given herd was modelled as the product of the herd-level true prevalence (HTP) and the within-herd prevalence conditional on the herd being infected (CWHP). HTP was modelled as a Bernoulli distribution with two possible outcomes; a herd was considered to be infected with probability μ and uninfected with a probability 1-μ. Then, conditional on the herd being infected, the conditional within-herd prevalence (CWHP) was modelled as beta distribution.

Prior distributions for HTP (μ) and CWHP were constructed as follows. In accordance to McAloon et al. (2016), a flat distribution from 0 to 1 was chosen as a prior for μ as there was no prior knowledge to inform a herd prevalence estimate. CWHP was based on an observational study on within-herd dynamics of *M. bovis* in five Dutch dairy herds with a clinical outbreak of *M. bovis* (Penterman et al., 2022). During the first three months after the onset of the outbreak in these herds, *M. bovis* DNA was found in conjunctival fluid in 34.3% of randomly selected healthy cattle and 65.9% of clinically suspect cattle, on average. The percentage of clinically suspected dairy cows per outbreak farm varied from 1% to more than 10%. Therefore, for our study a weighted within-herd prevalence was used, based on a 90% weight of the randomly selected cattle prevalence and a 10% weight of the clinically suspect cattle prevalence. The resulting 37.5% mean was used to fit a beta distribution using the 'PriorGen' package in R 4.2.1 (Kostoulas, 2018). Differences in HTP between the herd-level factors herd size, open/closed farming system and regions were tested. Numbers were assumed to be significantly different of each other when the 95% BPI of their difference did not include zero.

Sensitivity analysis of the selected priors for μ (HTP) and CWHP was conducted by analysing a number of alternatives. Next to the flat uniform distribution as prior for μ to estimate HTP, three alternative priors were used representing various levels of HTP. In a low HTP scenario, a beta prior with a mode of 0.25 was used as beta(3.88, 9.63). In a medium HTP scenario, a beta prior with a mode of 0.50 was used (beta(4.94, 4.94)). In a high HTP scenario a beta prior with a mode of 0.75 was used (beta(9.63, 3.88)). Finally, alternative priors for CWHP were specified as uninformative (beta(1,1)) and weakly informative by giving it a wider distributional spread (beta(9.35,15.58)).

The model was coded using OpenBUGS and was compiled with three sets of initial values. A burn-in period of 5,000 iterations was applied; conclusions were based on the next 15,000 iterations. Visual inspection of the time series trace plots and the Brooks-Gelman-Rubin diagnostic was used to assure that the chains had converged. Autocorrelation plots were examined visually to ensure there was no strong autocorrelation between the Monte Carlo samples. The model code for Model 2 can be found in supplementary file S3.

3. Results

3.1. Serology

A total of 7828 cattle from 451 dairy farms were sampled between 3 October and 31 December 2019. Five hundred and twenty-three samples were excluded from analysis due to violation of the sampling criteria (related to the age of the animals), resulting in the analysis of 7305 samples from 415 herds. From these herds, 100 herds had insufficient test results from calves ($3 \leq n < 6$) and/or insufficient test results from cows ($6 \leq n < 12$), yet these were kept in the data set. An overview of the test results for ELISA A is provided in Table 3. The majority of the cattle tested seronegative (95.4%). From the subset of 100 herds, 1799 samples were also tested for *M. bovis* specific antibodies using ELISA B, of which 1200 from cows and 599 from calves. The joint test results are shown in Table 4.

3.2. Test characteristics (Model 1)

Table 5 provides an overview of the posterior summary statistics for each Scenario. Animal-level prevalences of the populations are omitted as they are not in the scope of this model. In Scenario 1, posterior median Se and Sp estimates for ELISA A were 14.1% (BPI: 11.6–16.7) and 97.2% (BPI: 95.9–98.4), respectively (Table 5) (Fig. 1). For ELISA B, Se and Sp estimates were 92.5% (BPI: 88.3–96.5) and 99.3% (BPI: 98.7–99.8), respectively.

SeA was estimated to be 12.6% (BPI: 10.2–15.2) in Scenario 2 (with sera from cows only) but significantly higher in Scenario 3 (with sera from calves only: 36.3% (BPI: 27.5–44.9)). For ELISA B, SeB shifted slightly towards a median of 96.3% (BPI: 93.9–98.2) in Scenario 2 and to 90.8% (BPI: 85.6–95.3) in Scenario 3. SpA was estimated to be 96.6% (BPI: 94.7–98.3) in Scenario 2% and 99.3% (BPI: 98.0–100) in Scenario 3. SpB in Scenario 2 and Scenario 3 remained fairly the same as in Scenario 1 (Table 5 and Fig. 1).

In the subset of herds with a clinical outbreak (Scenario 4), SeA increased considerably to a median of 70.7% (BPI: 63.8–77.6) and SeB shifted to 94.3% (BPI: 91.0–97.2). SpA and SpB remained fairly the same as in Scenario 1 (Fig. 1).

Covariances between the tests were estimated at 0.004 (BPI: −0.01 to 0.013) in diseased subjects and 0.001 (BPI: −0.000 to 0.005) in non-diseased subjects in Scenario 1. These estimates did not shift substantially in the other scenarios. The potential scale reduction factor was between 0.99 and 1.00 for all parameters in each scenario and trace plots were stable (results not shown), indicating proper convergence. Effective sample sizes did not reveal problematic autocorrelation.

The alternative priors selected for Scenario 1 yielded adequate model convergence and effective sample size values. Inspection of the posterior distributions showed that the posterior of SeA and SeB shifted to lower values under uninformative and weakly informative priors, suggesting the original prior specification had an impact on the results (Fig. 2). Posterior distributions of SpA and SpB were very similar across the selected prior distributions, implying robustness across different prior settings.

3.3. Estimated prevalence (Model 2)

The regional location of herds in the study population was representative for the distribution of dairy herds in the Netherlands, i.e. most herds located in the northern and eastern region (Table 6). The mean herd size was 124 cows. Over 55% of the herds had a closed farming system, which is higher than the national average in dairy farms in 2019 (48.2%; Government of the Netherlands, 2020). Herds from the northern region where largest and had most often a closed farming system.

Posterior median cow-level true prevalence (π) was 26.3% (BPI: 23.5–29.1) (Table 7). The posterior median herd-level true prevalence (HTP) was 69.9% (BPI: 62.7–77.6). It was observed that the model was very insensitive to the prior for μ (HTP), therefore only results of the default model with a flat distribution from 0 to 1 as the prior for μ are shown. Changing the CWHP prior to an uninformative or weakly informative distribution lead to moderate shifts in posterior median HTP toward 81.4% (BPI: 73.3–89.2) and 72.5% (65.1–80.2), respectively.

HTP was not statistically different between regions. Herds with an

Table 3
M. bovis-specific ELISA (A) results of 2346 calves and 4959 cows from 415 dairy herds in the Netherlands in 2019.

Test result	Calves	Cows	Total
0	2272 (97%)	4699 (95%)	95.4%
+	34 (1%)	197 (4%)	3.2%
++	26 (1%)	45 (1%)	1.0%
+++	10 (0.4%)	15 (0.3%)	0.3%
++++	1 (<0.1%)	2 (<0.1%)	< 0.1%
+++++	3 (0.1%)	1 (<0.1%)	< 0.1%
Total	2346	4959	7305

open farming system had a probability of infection of 74.3% (BPI: 66.4–82.6) and herds with a closed farming system had a probability of infection of 67.0% (BPI: 58.7–75.7) (Table 7). The difference between these types of farming systems was significantly different from zero (median: +7.2% (BPI: 0.2–14.2)). Herds belonging to the group of 25% largest herds had a higher probability of infection (77.7% (BPI: 70.5–85.6)) than herds belonging to the group of 25% larger herds (64.2% (BPI: 55.3–74.8)), with a median difference of + 13.3% (BPI: 5.0–21.8). Herds belonging to the group of 25% largest herds also had a higher probability of infection than herds belonging to the group of 25% smaller herds (65.6% (BPI: 55.2–77.1)), with a median difference of + 12% (BPI: 2.5–21.4). The posterior distribution of the animal-level TP and herd-level TP is shown in Fig. 3.

The variation in Se estimates between ELISA A and ELISA B is reflected in the probability of a herd being infected despite having no positive test results. From the 315 herds investigated with ELISA A, 194 had no positive test results. In these herds, the estimated probability of the herd being infected ranged from 37% to 52% (Fig. 4). From the 100 herds investigated with ELISA B, 15 had no positive test results and the probability of these herds being infected did not exceed 1% (Fig. 4).

4. Discussion

In this study, test characteristics of two antibody ELISAs were validated under field conditions. As no gold standard was available for *M. bovis* infections on a herd level, Bayesian modelling was used to estimate test sensitivities and specificities and to estimate the prevalence of *M. bovis* infected Dutch dairy herds.

A latent class model was used for two tests in 100 populations, assuming conditional dependence of the test results. This assumption appeared appropriate as the model estimated some (yet limited) level of covariance between diseased subjects. By considering the 100 herds as subpopulations, the assumption of differences in the prevalence of infection among sampled populations, when applying a latent class model to data from two tests (Hui and Walter, 1980), was met. Also, by doing so, test accuracies were estimated under realistic (field) conditions, reflecting test error rates for cattle randomly selected from the target population as a whole (Hanson et al., 2003). However, there is also a downside of differing prevalences amongst subpopulations. The assumption of constant accuracy of tests across populations may have been violated, as test sensitivity might be biased toward the value in the population(s) with the largest prevalence (Toft et al., 2005). We assume that giving the large number of randomly selected herds, this bias has been limited.

Test results of the two ELISAs were dichotomized prior to analysis, based on the cut-off S/P ratio as defined by the manufacturer. By doing so, all positive test results were considered equal, which may have led to some loss of information (Kostoulas et al., 2017). A latent class model based on continuous responses of the two ELISA tests under evaluation may have been able to quantify antibody level-dependent sensitivities more accurately (for example related to the stage of disease).

Assessing how robust (or not) model results are to different prior settings is an important part of Bayesian statistics (Kostoulas et al., 2017; Depaoli et al., 2020). Therefore, Model 1 was rerun with two alternative specifications for the priors for sensitivity and specificity. This revealed that the original prior specification for the sensitivities of the two ELISA tests had a considerable impact on the results. An explanation for this dependency could lie in the source of the informative priors selected, which were based on calf serum samples, whereas our data predominantly comprised results of serum samples from cows. Cattle with a higher age may have been infected for a longer duration of time, which may have led to waning antibody levels. Contrary, seropositivity in young calves is probably the result of recent infection, although maternal antibodies cannot be excluded. If the ELISAs we evaluated suffer from antibody level-dependent sensitivity, or a very short duration of antibody detection as suggested by Petersen et al. (2020), then the informative priors for sensitivities we selected based on studies in calves may have been too high for our data. Nevertheless, the substantive interpretation of model results did not change under different prior specifications.

Table 4
Cross-classification of M. bovis results from two correlated ELISA tests in serum samples from calves and cows (N = 1799).

	ELISA B					
ELISA A	0	+	+ +	+ ++	+ ++ +	Total
0	1025	91	130	110	310	1666 (92.4%)
+	19	5	16	12	35	87 (5.0%)
+ +	11	3	2	4	11	31 (1.7%)
+ ++	1	1	0	0	9	11 (0.7%)
+ ++ +	0	0	0	0	2	2 (0.1%)
+ ++ ++	0	1	0	0	1	2 (0.1%)
Total	1.056 (58.8%)	101 (5.8%)	148 (8.2%)	126 (7.0%)	368 (18.3%)	1799

Table 5
Posterior medians and 95% posterior probability intervals of the animal-level prevalence π and test characteristics using Model 1 on all joint test results from 100 herds (Scenario 1), data from cows only (Scenario 2), data from calves only (Scenario 3) and joint test results of 5 herds with a high prevalence (Scenario 4).

	Scenario 1: Total		*Scenario 2:* Cows only		*Scenario 3:* Calves only		*Scenario 4:* High prevalence	
Parameter	Median	95% BPI	Median	95% BPI	Median	95% BPI	Median	95% BPI
SeA	0.141	0.116; 0.167	0.126	0.102; 0.152	0.363	0.275; 0.449	0.707	0.638; 0.776
SeB	0.925	0.883; 0.965	0.963	0.939; 0.982	0.908	0.856; 0.953	0.943	0.910; 0.972
SpA	0.972	0.959; 0.984	0.966	0.947; 0.983	0.993	0.980; 1.000	0.965	0.872; 1.000
SpB	0.993	0.987; 0.998	0.993	0.985; 0.998	0.995	0.989; 0.998	0.991	0.981; 0.997
covp	0.004	-0.008; 0.013	-0.002	-0.014; 0.006	-0.021	-0.055; 0.018	0.009	-0.015; 0.036
covn	0.001	-0.000; 0.005	0.002	-0.000; 0.007	0.002	-0.000; 0.006	0.003	-0.001; 0.011

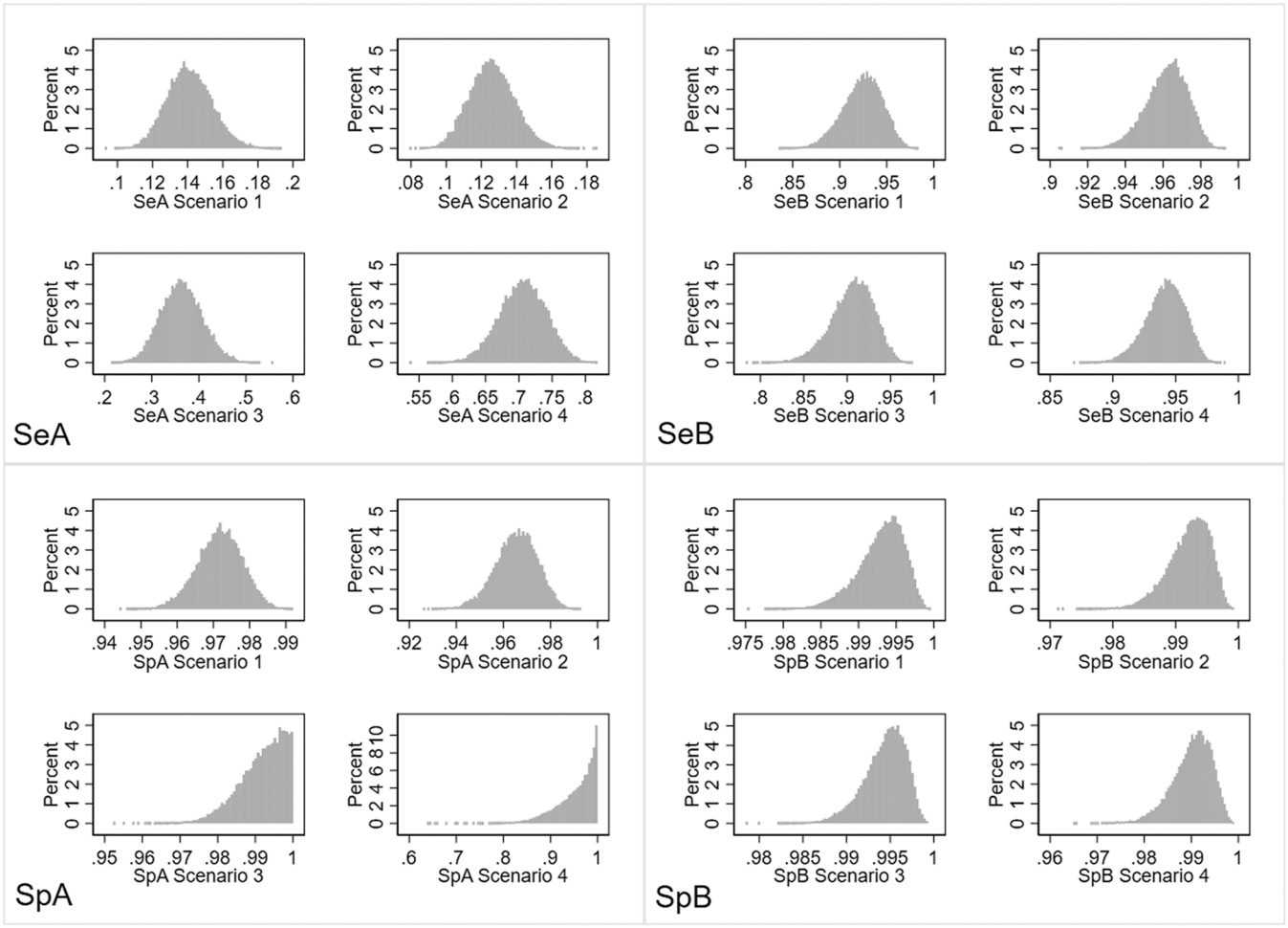

Fig. 1. Posterior distributions of Se and Sp of ELISA A and ELISA B from four latent class models: all serum samples (Scenario 1), cows only (Scenario 2), calves only (Scenario 3) and high-prevalent herds (Scenario 4).

Results showed that the sensitivity of commercially available ELISA kits to identify antibodies against *M. bovis* vary largely. The low sensitivity of the Bio-X K-260 ELISA has also been shown by others (Andersson et al., 2019; Petersen et al., 2020), although they used the monowell K-302 ELISA in their studies instead of the double well K-260 ELISA from Bio-X, with both antigen-coated wells and negative control antigen-coated wells. The K-260 ELISA was used for routine diagnostics at Royal GD at the time of this study. Wawegama et al. (2016) estimated the sensitivity of the Bio-X K-260 ELISA to be 13% (95% CI: 5–30), which is in line with our findings in the default scenario (i.e. cows and calves from randomly chosen herds). The low sensitivity of the Bio-X ELISA needs to be taken into account when used in the field. Suggested implications for the poor sensitivity of the corresponding Bio-X K-302 ELISA are a very short duration of antibody detection and its ability to primarily detect clinically ill animals (Petersen et al., 2020). The latter seems to be true for the Bio-X K-260 ELISA as well, as it's sensitivity improved considerably to 70.7% (BPI: 63.8–77.6) when samples from herds with a recent clinical *M. bovis* outbreak were analysed.

The aforementioned hypothesis of reduced sensitivity when antibody levels decrease is supported by our finding that Bio-X test sensitivity was twice as high in samples from calves as compared to samples from cows. The sensitivity of the IDvet ELISA was estimated to be 92.5%, with no notable differences between samples from calves, cows or herds with a clinical outbreak. This suggests high sensitivity to detect both recent and past infections. Nevertheless, this raises questions to the interpretation of IDvet ELISA results when used in practice in relation to the purpose of testing. It is hypothesized that the IDvet ELISA in serum will measure (past) exposure to *M. bovis* rather than current colonization in the infected animal only (Petersen et al., 2020), which has to be taken into account when used for diagnostic purposes without parallel pathogen detection. More importantly, the duration of serum antibody response after natural infection is key in this matter, which is not exactly known. Vähänikkilä et al. (2019) measured serum antibodies for at least one and a half years in cattle from farms with and without apparent presence of *M. bovis*. On the contrary, Petersen et al. (2018) showed that serum antibody responses are highly dynamic and show a high level of variation between individual cows.

In our attempt to estimate the true prevalence of dairy herds with an infection of *M. bovis*, test results of the two aforementioned ELISA tests were considered simultaneously in one model, using either test results from the Bio-X ELISA ($n = 315$ herds) or from the IDvet ELISA ($n = 100$ herds). A latent class model in which the available cross-classified test results were used did not converge as 315 out of 415 herds lacked IDvet ELISA results. It would be worthwhile to investigate other models or software packages than the one we used to solve this matter. Also, ELISA test characteristics were not taken into account in the sample size calculation for the prevalence estimation, which has most likely led to an underestimation of the required sample size considering the suboptimal test sensitivities of both ELISAs.

Herd-level true prevalence was estimated to be high, suggesting that a large proportion of the dairy herds in the Netherlands have been exposed to *M. bovis*. However the Bayesian latent-class model that we used tends to overestimate true herd prevalence (McAloon et al., 2019).

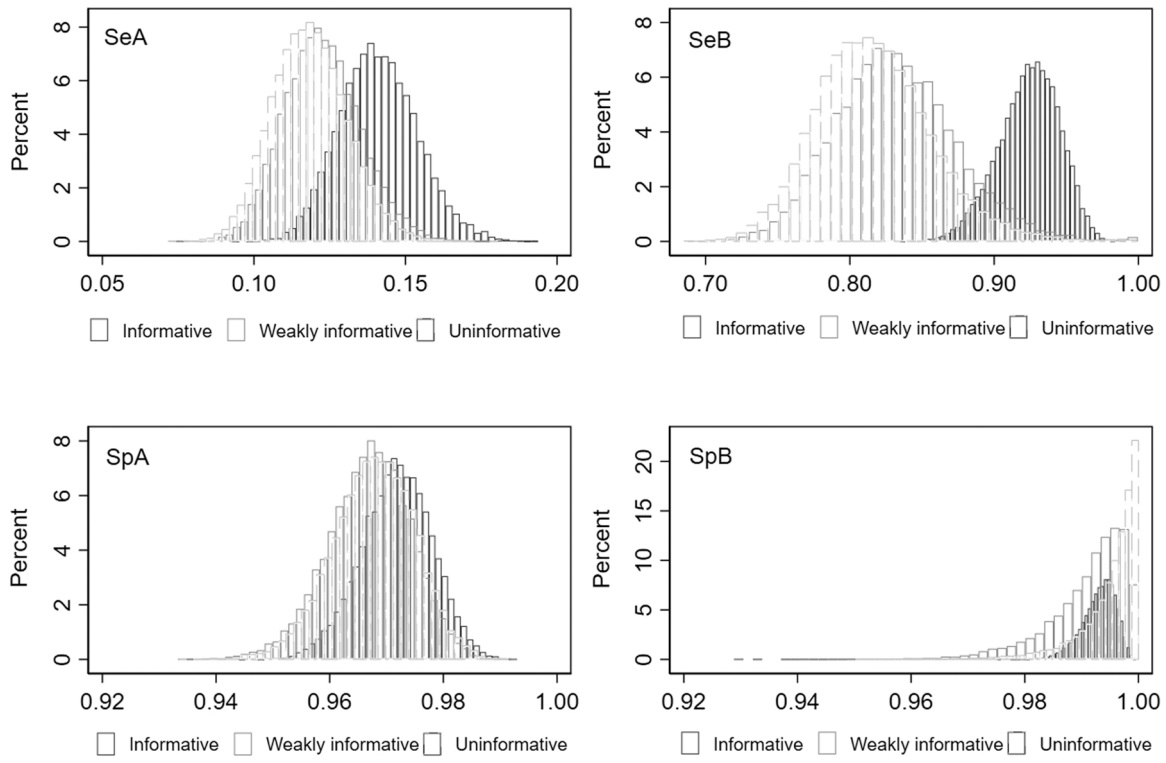

Fig. 2. Posterior distributions of Se and Sp of ELISA A and ELISA B under informative (dark grey), weakly informative (medium grey) and uninformative (dashed) prior settings in the default latent class model with two tests and 100 populations.

Table 6
Descriptive statistics of the investigated herds (N = 415).

Region	Number of herds	Mean herd size[a] (SD)	Closed farming system[b] (%)	Test positive herds (≥1 T^+ animal, %)
North	121	138 (79)	61.2	42.3
East	138	115 (52)	53.2	50.0
West	73	107 (47)	49.3	42.2
South	83	131 (62)	56.6	50.8
Total	415	124 (63)	55.5	44.0

[a] Number of lactating cows in third quarter of 2019
[b] No introduction of new animals in the herd in the past year in the third quarter of 2019

Table 7
Posterior medians and 95% posterior probability intervals of the *M. bovis* prevalence parameters of Model 2. Parameters sharing an alphabetical superscript have a difference being significantly different from zero.

Parameter	Median	95% BPI
Cow-level true prevalence	0.263	0.235; 0.291
Herd-level TP	0.699	0.627; 0.776
Herd-level TP in North	0.653	0.562; 0.752
Herd-level TP in East	0.732	0.652; 0.819
Herd-level TP in West	0.671	0.562; 0.781
Herd-level TP in South	0.735	0.639; 0.831
Herd-level TP in closed farming systems[#]	0.670[a]	0.587; 0.757
Herd-level TP in semi-open farming systems	0.689	0.533; 0.833
Herd-level TP in open farming systems	0.743[a]	0.664; 0.826
Herd-level TP in 25% smallest herds[$]	0.684	0.561; 0.789
Herd-level TP in 25% smaller herds	0.656[b]	0.552; 0.771
Herd-level TP in 25% larger herds	0.642	0.553; 0.748
Herd-level TP in 25% largest herds	0.777[b]	0.705; 0.856

#Closed: 0 cattle introduced in the herd in the past year. Semi-open: 1–2 cattle introduced in the herd in the past year. Open: > 2 cattle introduced in the herd in the past year. Both measured in the third quarter of 2019.
$25% smallest: < 68 lactating cattle; 25% smaller: 68–96 cattle; 25% larger: 97–131 cattle; 25% largest: > 131 cattle.

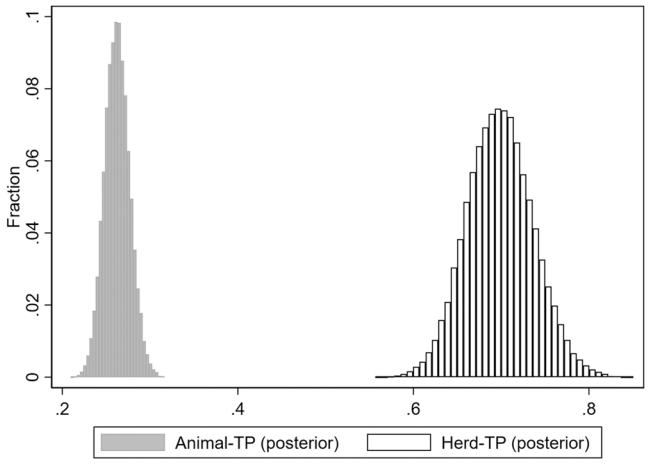

Fig. 3. Posterior distributions of animal-level true prevalence (grey) and herd-level true prevalence (white).

More specifically, it was concluded that the model is quite sensitive to the herd true prevalence (HTP) prior used. We therefore varied the prior for HTP from an uninformative uniform distribution from 0 to 1 to informative beta distributions representing a low, medium or high HTP. The resulting posterior HTP estimates changed only marginally with varying HTP priors, suggesting that our model was robust to the selection of the prior for HTP.

For the within-herd prevalence (CWHP), a beta prior distribution was chosen based on observational study results in dairy cattle herds with an *M. bovis* outbreak (Penterman et al., 2022). This type of prior was chosen as it resulted in the most accurate model results and therefore appeared to be most appropriate according to McAloon et al. (2019). Moreover, McAloon et al. (2019) concluded that a Bayesian latent-class model can be reasonably accurate when used to estimate

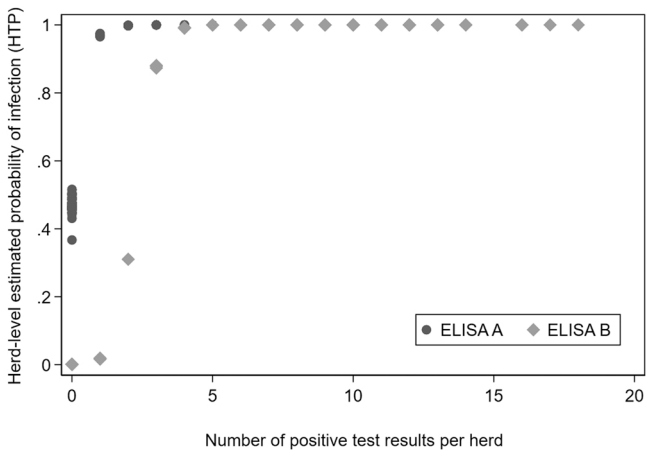

Fig. 4. Posterior herd-level probability of infection and the number of positive ELISA test results per herd. Herds investigated with ELISA A as shown as dots (N = 315) and herds investigated with ELISA B are shown as diamonds (N = 100).

prevalence for infections or diseases with poor Se *or* low CWHP (but not both). Indeed, poor Se may be an issue when estimating *M. bovis* prevalence, as shown with Model 1 in this study, depending on the ELISA kit used. Yet it is expected that *M. bovis* within-herd prevalence in infected herds is fairly high, as *M. bovis* DNA was found in conjunctival fluid in 34.3% of healthy cattle in herds with an acute outbreak (Penterman et al., 2022). Changing the CWHP prior to an uninformative or weakly informative distribution led to an increase in posterior *M. bovis* herd prevalence estimated by our model. Even though the posterior BPIs of these alternatives overlapped with the BPI under the original prior, it does suggest that the CWHP we used (based on Penterman et al., 2022) may be an underestimation of the true within-herd prevalence in infected herds.

No statistically significant differences were found in true prevalences between regions. Large dairy herds and herds that introduced cattle from other herds had a higher probability of being infected than smaller dairy farms or closed farms. This is in agreement with previous studies, in which herd size has been identified as a risk factor for the detection of *M. bovis* in bulk milk (Fox et al., 2003; Pinho et al., 2013; Vähänikkilä et al., 2019; McAloon et al., 2022; Hurri et al., 2022), although it has also been described to be not associated with the bulk milk antibody test result (Petersen et al., 2016). Purchase of a carrier animal has also been described as an important risk factor (Maunsell et al., 2011; McAloon et al., 2022).

It is unknown which proportion of our sample of dairy herds have experienced clinical signs of *M. bovis*-associated disease. Moreover, the clinical relevance of seropositive test results in herds without clinical signs requires further clarification, as it has been suggested that *M. bovis* ELISAs may cross-react with commensal *Mycoplasmas* (Wawegama et al., 2014; Petersen et al., 2020). In the light of the difference in sensitivity of the ELISAs used in our study, and the aforementioned effect of recent versus past infections, it is expected that true herd prevalence of 69.9% represents farms with recent exposure to *M. bovis* as well as farms with past exposure to *M. bovis*. These results suggest that *M. bovis* infection is endemic in the Dutch dairy sector. Further research is needed to identify the number of active infections in the dairy sector.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. Veldhuis reports a relationship with Royal GD that includes: employment. M. Aalberts reports a relationship with Royal GD that includes: employment. P. Penterman reports a relationship with Royal GD that includes: employment. G. van Schaik reports a relationship with Royal GD that includes: employment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2023.105946.

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