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A longitudinal study of the dynamics of *Mycoplasma bovis* antibody status in primiparous cows and bulk tank milk in Swedish dairy herds

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ABSTRACT

Mycoplasma bovis is an important pathogen causing pneumonia, mastitis, and arthritis in cattle all over the world entailing reduced animal welfare and economic losses. In this longitudinal study, we investigated the presence of *M. bovis* antibodies in bulk tank milk (BTM) and in milk from primiparous (PP) cows at 4 sampling occasions over 2 yr. Herd characteristics associated with a positive antibody test result in PP cows were investigated. The participating dairy herds (n = 149) were situated in southern Sweden, samples were collected and analyzed with ID Screen antibody ELISA. Information on herd characteristics was retrieved from the national DHI database. To identify herd characteristics associated with the presence of antibodies in PP cows, mixed linear regression with herd and sample as random factors were used. The apparent herd-level prevalence of *M. bovis* infection based on antibodies in BTM was 17%, but with the addition of PP cows, the prevalence increased to 28%. The results showed that larger herds and introduction of cattle was associated with higher antibody levels in PP cows. In conclusion, this study showed a clear difference in the apparent prevalence of *M. bovis* infection based on antibodies in BTM or in PP cows: The number of positive herds was almost doubled when including PP cows. This motivates repeated sampling of a few PP cows to find newly infected herds in an early stage. Finally, the results showed that introduction of cattle influences the level of *M. bovis* antibodies. This is important in the control and prevention of further spread of the infection. It is essential for free herds to know their *M. bovis* status, and antibody testing is highly recommended if introducing cattle.

Key words: heifers, ELISA, *Mycoplasmopsis bovis*, external biosecurity

INTRODUCTION

Mycoplasma bovis causes disease in cattle, with pneumonia, arthritis, and mastitis being the most common clinical presentations (Nicholas and Ayling, 2003; Maunsell et al., 2011). These infections often become chronic and respond poorly to antibiotic treatment, leading to economic losses for the farmers and reduced animal welfare (Maunsell et al., 2011). *Mycoplasma bovis* was first diagnosed in the United States in 1961 (Hale et al., 1962) and has since been detected in cattle all over the world (Nicholas and Ayling, 2003). During the last decade, the importance of *M. bovis* has escalated due to introduction in countries that were previously free, outbreaks in major dairy-producing countries, and the report of a new strain spreading in the Northern European countries (Pothmann et al., 2015; Haapala et al., 2018; Tardy et al., 2020). The most recent introductions were reported in Sweden in 2011, in Finland in 2012 and in New Zealand in 2017 (Dudek et al., 2020; Jordan et al., 2021; Hurri et al., 2022). *Mycoplasma bovis* can cause serious illness, and it may lead to increased use of antimicrobial drugs and increased risk of resistance to several antimicrobial drugs (Gautier-Bouchardon et al., 2014; Klein et al., 2019). There have been numerous efforts to develop efficacious vaccines against *M. bovis* but without success (Perez-Casal et al., 2017). Therefore, there is a need to control and prevent the infection from spreading to new herds and to be able to detect infected cattle, especially subclinically infected animals (Caswell and Archambault, 2007).

Diagnosing *M. bovis* on cattle has historically been done by culture, and later PCR, on milk or nasal swabs, but due to the intermittent shedding of *M. bovis*, serology could be a better option or a supplementary method (Park-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

er et al., 2017). Asymptomatic carriers are a diagnostic challenge, and analyzing antibodies could be a method to identify such animals (Maunsell et al., 2011). Evaluation of commercial *M. bovis* antibody ELISA has been done in recent studies using Bayesian latent class analysis. In these studies, the IDvet ELISA (ID Screen *Mycoplasma bovis* Indirect, Grabels, France) showed a high sensitivity (92.5%–94%) and specificity (92%–99.3%; Andersson et al., 2019; Veldhuis et al., 2023; McAloon et al., 2024). Several studies have been investigating the antibody dynamics and course of *M. bovis* infection in dairy herds using ELISA, to detect antibodies (Vähänikkilä et al., 2019; McCarthy et al., 2021; Penterman et al., 2022). To identify herds that have been exposed to *M. bovis*, herd-level screening is needed; antibodies in bulk tank milk (**BTM**) or milk from individual animals could be a useful tool for this purpose (Petersen et al., 2016). The duration of antibodies is not well understood. In a Finnish study, some animals remained seropositive to *M. bovis* for at least 1.5 yr after the index case, regardless of clinical symptoms of *M. bovis* infection being present on the farm (Vähänikkilä et al., 2019). The antibody response in individual cows was dynamic and varied a lot between cows in a Danish study (Petersen et al., 2018).

There is a need for increased knowledge about *M. bovis* prevalence and optimal diagnostic strategy in dairy herds to effectively prevent and control the disease. Testing a small sample of primiparous (**PP**) cows has been found useful in monitoring herd infection status for other infections, such as bovine respiratory syncytial virus and bovine coronavirus (Ohlson et al., 2013). In Sweden, the prevalence of *M. bovis* at herd level was 4.8% in a national screening of antibodies in BTM performed in 2019, with large regional differences ranging from 0 to 20%, with a higher prevalence in the south of Sweden (3%–20%; Hurri et al., 2022). Since the first cases in 2011, *M. bovis* has spread in dairy herds and fattening herds in the south of Sweden, symptoms being primarily pneumonia and arthritis in feedlot calves, but very few cases of mastitis in dairy cows (Hurri et al., 2021).

Recent studies have identified purchase of cattle (Schibrowski et al., 2018; Murai and Higuchi, 2019; Fujimoto et al., 2020) and the use of a breeding bull (Gille et al., 2018) as risk factors for introduction of *M. bovis* into dairy herds. Other studies have also concluded that large herd size is a risk factor for *M. bovis* infection (Thomas et al., 1981; Haapala et al., 2021; Hurri et al., 2022). The costs of introducing *M. bovis* disease in the herd include production losses, veterinary costs, labor for treatment and care of sick animals, mortality, premature culling, and costs for implementing diagnostic and control measures (Maunsell et al., 2011). Dairy cows infected with *M. bovis* showed higher SCC, produced less milk, and had lower milk fat and urea content compared with *M. bovis*-negative dairy cows in a study from Estonia (Timonen et al., 2017). However, BTM SCC were not higher in *M. bovis*-positive herds compared with negative herds in a study in United States (Fox et al., 2003). The effects and costs associated with calf respiratory disease include reduced fertility, increased age at first calving, and reduced milk production later in life (Maunsell and Donovan, 2009). A recent cross-sectional study by Hurri et al. (2022) showed that there was a higher late calf (2–6 mo of age) and young stock (6–15 mo of age) mortality in herds with seropositive BTM. Effects on animal health and performance need to be further investigated by following dairy herds over time.

In this study, the aims were (1) to monitor the changes in *M. bovis* antibody status in BTM and milk from PP cows in Swedish dairy herds over time, and (2) to investigate potential risk factors and herd health variables associated with *M. bovis* antibodies in primiparous cows.

MATERIALS AND METHODS

Study Population

Five regions (Halland, Kalmar, Skåne, Västra Götaland, and Östergötland) in the south of Sweden with previously known cases of *M. bovis* were selected for the study. Dairy herds in these regions with an average of more than 70 cows (both lactating and dry) on a yearly basis and affiliated with the DHI program (Växa Sverige) were invited to participate in the study. A list of herds (n = 976) fulfilling these selection criteria was retrieved from the dairy farmers' association, Växa Sverige, and all were invited by mail. Out of these, 139 herds (14%) agreed to participate in the study. Another 10 herds joined later after contact with participating herds or due to *M. bovis* being detected on the farm. There were 4 herds that stopped delivering milk during the study and therefore dropped out.

The DHI database collects and stores cow data from monthly milk testing, disease treatments, and production parameters. Around 70% of the dairy herds in Sweden comprising 77% of the cows were affiliated eith the DHI program year September 1, 2019 to August 31, 2020 (Cattle Statistics 2021, Växa Sverige, 2021). In our study, 87% of the herds (n = 130) were available in the DHI database; the other herds had withdrawn from the DHI program after the start of the study.

Sampling Procedures

Herds were sampled 4 times at ~6-mo intervals, in (1) September 2019, (2) February 2020, (3) November 2020, and (4) May 2021, with a 2-mo long sampling window each time (Figure 1). For each herd, on each sampling

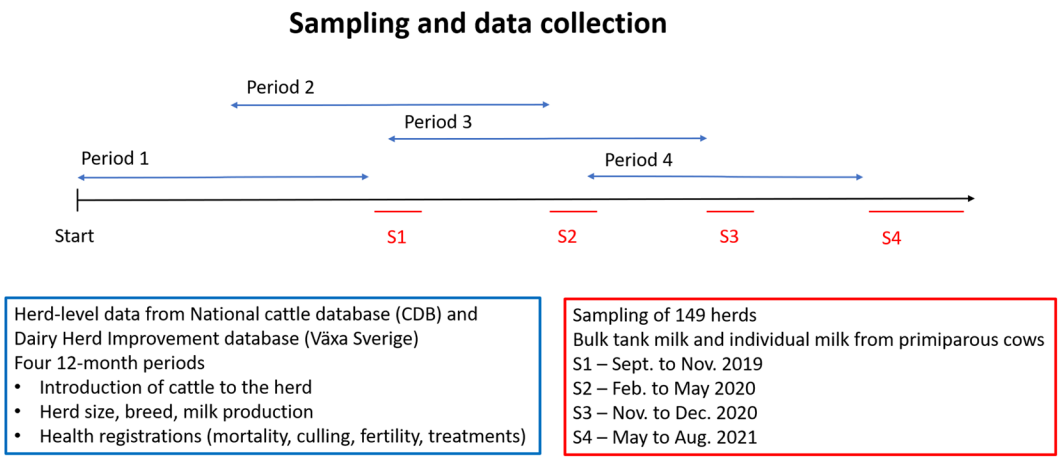

Figure 1. Longitudinal study with sampling of 149 herds 4 times (S1–S4) with approximately 6 mo intervals for 2 yr. Herd-level data retrieved in 12-mo periods (period 1–4) before the start of the sampling.

occasion, milk samples were collected from the 3 youngest home-bred PP cows and BTM. The samples were collected at the milk testing laboratory (Eurofins Steins Laboratory, Jönköping, Sweden), in conjunction with the routine milk quality analysis. Between 0 and 9 PP cows were sampled from each herd, depending on available PP cows at the monthly test milking. To be included in the primiparous sampling, the cows had to be within 6 mo after calving. The samples were collected in 10-mL test tubes containing 1.5 mg of the preservative agent Bronopol (2-bromo-2-nitropropane-1,3-diol) and sent by postal service in ambient temperature to the Swedish University of Agricultural Sciences (**SLU**), Uppsala, Sweden. The samples were stored at −20°C until analysis.

There were 19 herds not affiliated with the DHI database, and from these herds, samples from PP cows could not be collected automatically from the milk testing laboratory. In addition, in 19 herds no PP cows were test milked during the sampling window. Therefore, sample kits were sent out to these farmers to collect and submit milk samples from PP cows. These samples were received at the laboratory between February 2020 and August 2021. Not all herds submitted samples, and in total, 10% to 20% (n = 14–31) of the herds lacked PP samples at one or more sampling occasions.

Milk Analysis

The samples were analyzed for IgG antibodies to *M. bovis* with ID screen indirect ELISA, (IDvet, Grabels, France) at the Department of Clinical Sciences, SLU, in accordance with the manufacturer's instructions. The relative amount of antibodies in the samples was calculated as [sample optical density (OD) − negative control OD]/(positive control OD − negative control OD) × 100 (S/P%). The milk samples were analyzed with the overnight incubation protocol and the cut-off S/P% ≥30% was applied.

Assessment of Infection Dynamics on Herd Level

Herd-level *M. bovis* antibody status was defined as 1 of the 6 following categories at each sampling occasion: (1) BTM and milk from PP cows negative, (2) BTM negative and PP missing, (3) BTM negative and PP positive, (4) BTM and PP positive, (5) BTM positive and PP missing or (6) BTM positive and PP negative. If at least one PP cow was positive, the herd was categorized as PP positive.

Bulk tank milk antibody level was compared with the number of positive PP cows (0, 1, 2, or 3), for all sampling occasions. Herds with more than 3 sampled PP cows (n = 199) were transferred to one of the other categories by dividing the number of positive PP cows with the total number of PP cows. Herds with less than 3 PP cows sampled (n = 13) were included in group 0, 1. or 2. Student's *t*-test was used to evaluate the correlation between the number of antibody-positive PP cows and the antibody level (S/P%) in BTM.

Data

Herd-level data on health and production variables were retrieved from the DHI database. The data were aggregated in four 12-mo periods calculated backward from the start of each sampling period: (1) September 1, 2019, (2) February 1, 2020, (3) November 1, 2020, and (4) May 1, 2021 (Figure 1). Data regarding mortality, culling rates, reproductive performance, and veterinary-reported clinical diseases were calculated as

cases per 100 animals at risk. Herd size was calculated as the average number of cows (both lactating and dry) over the 12 mo. Milk production was calculated as the mean production per cow in kilograms of ECM for the 12-mo period. Bulk tank milk SCC in thousands of cells/mL was calculated as the arithmetic mean of 12 monthly measurements. Breed was classified into 4 categories on herd level, the main breed constituting more than 80% of the cows. Distribution of breed was Swedish Holstein (**SH**) 45% of the herds, Swedish Red (**SR**) 15%, mixed SH and SR 28% and other breeds 12%. SR and SH are the 2 main dairy cow breeds in Sweden. Herd-level data in 12-mo periods regarding herd size (i.e., number of female animals >24 mo old), number of introduced cattle, and number of herds that the introduced cattle originated from (**INHERDS**) were collected at the Swedish Board of Agriculture covering the period from 2018-09-01 to 2021-08-31 (Swedish Board of Agriculture, https://jordbruksverket.se/). Introduced cattle includes all cattle purchased to the farm and the farm's own cattle returning from another external farm or from pasture, located more than 500 m away from the main farm. The data from the National Cattle Database were aggregated in 12-mo periods before the exact date of sampling of the PP cows, but if the sampling date was missing, the period 12 mo before the start of each sampling period was used. Data on herd size (number of milking cows) in 15 herds not affiliated with the DHI were retrieved from a questionnaire answered by these farmers during the fall 2020. In Figure 1, the data collection and sampling periods are visualized.

Statistical Analysis of Risk Factors

The risk factor analysis assessed the effects of herd size and introduction of cattle on *M. bovis* antibody status in PP cows. The analysis also included predictors such as region, breed, INHERDS, median age of the introduced cattle, and antibody level in BTM. We used individual antibody ELISA test results from PP milk samples, (S/P%), as the continuous outcome variable in the model. The outcome variable was log-transformed to achieve normal distribution of the residuals. Each of the herd-level variables was first evaluated univariably in a linear mixed regression model with herd and sample as random effects. A threshold of $P \leq 0.20$ was chosen for detecting potential risk factors to be included into the multivariable models. A manual backward stepwise elimination was used to exclude nonsignificant ($P > 0.05$) variables from the multivariable model, to find the reduced model that best explained the data. At each step the variable with the highest P-value was removed and when all remaining variables had a P-value < 0.05 the regression model was final. After omitting a variable, previously omitted variables were tested again, and the model was re-examined. This was possible because we had a limited number of variables in the full model. Model evaluation was assessed with normal probability plots, and plots of residuals versus the predicted values were constructed and evaluated for outliers.

Statistical Analysis of Health and Production Variables

The statistical analysis assessed if the *M. bovis* antibody status at herd level was predictive of the various herd-level production and health variables. For the *M. bovis* status, 3 categories were possible: (1) negative in both BTM and PP cows, (2) negative BTM and positive PP cows, or (3) positive BTM and positive, negative, or missing PP cows. Each of the herd variables was first evaluated univariably in a multilevel mixed-effects negative binomial regression with herd and sample as random effects. Variables with $P \leq 0.20$ were further analyzed in multivariable multilevel mixed-effects negative binomial regression model for each variable, correcting for biologically plausible variables such as herd size, introduction of cattle (yes or no), milk production, and breed, when adequate. A complete description of the variables offered to each regression model is available in Supplemental Table S1 (see Notes). All statistical analyses were performed using Stata (release 15.1; StataCorp LP, College Station, TX).

RESULTS

Antibody Prevalence at Herd Level

At start, there were 139 farms participating in the study, but due to dropouts (n = 4) and new farms (n = 10) added in the study period, there was a maximum of 145 sampled farms at a single sampling occasion (Table 1). The median herd size was 150 cows (interquartile range [**IQR**] = 87–247).

Geographical Distribution of Herds

The distribution of participating herds and their *M. bovis* antibody status in BTM and milk from PP cows, including changes in status, is presented in Figure 2. Change in status was defined from sampling 1, herds negative at sampling 1 and positive in either sampling 2, 3, or 4, were considered new positive herds. All the herds (n = 10) that joined the study after sampling 1 had the same status the whole period, 4 were negative and 6 were positive both in BTM and PP cows. One-third of the participating herds were situated in Västra Götaland and this region also had the highest number of new positive

Table 1. Antibodies to *M. bovis* in samples collected from bulk tank milk (BTM) and from primiparous (PP) cows on herd-level[1]

Sampling occasion	Herds with BTM samples, n	Herds with positive BTM, n (%)	Herds with BTM and PP samples, n	Herds with positive PP, n (%)	Herds with positive PP and negative BTM, n	Herds with negative PP and positive BTM, n
1	139	22 (15.8)	108	31 (28.7)	15	1
2	144	24 (16.6)	130	31 (23.8)	11	4
3	145	25 (17.2)	126	40 (31.7)	16	1
4	145	26 (17.9)	123	36 (29.3)	12	1

[1]PP positive herds had at least one antibody-positive PP cow.

herds. The highest number of BTM positive herds were situated in the southeast of Skåne.

Presence of *M. bovis* Antibodies on the Farms

In total, 18% (450/2,448) of the PP samples were positive, and the individual values for the positive cows varied between 30.2 and 354.5 S/P%. For the BTM, 17% (98/575) of the samples were positive, and the different values for the positive samples varied between 30.8 and 156.4 S/P%. The number of antibody-positive PP cows in each herd was significantly correlated with the antibody level (S/P%) in BTM ($P < 0.001$, Student's t-test, Figure 3). For all 4 sampling occasions, there were in total 487 samplings with test results from both BTM and PP cows.

The results for the analysis of BTM and PP samples at each sampling occasion are shown in Figure 4. At the study commencement 76 herds out of 139 were negative in both BTM and PP samples, 15 herds were negative in BTM and positive in PP sample, and 16 were positive in both sample types, 31 herds had only BTM samples (26 negative and 5 positive). Considering a herd positive at a sampling occasion either on positive BTM or at least

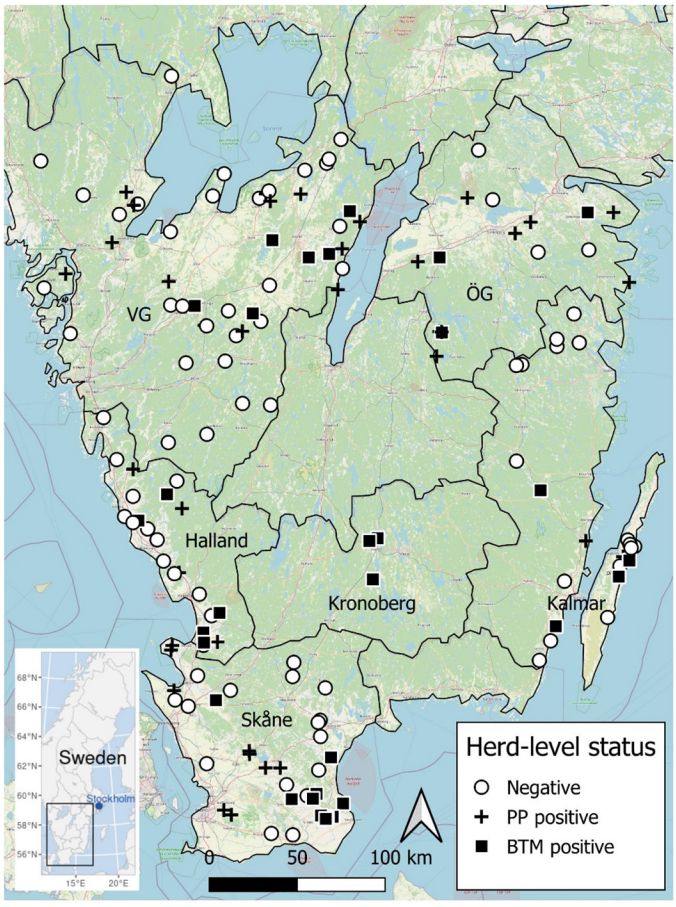

Figure 2. Geographical distribution of the participating herds, stratified by antibody status to *Mycoplasma bovis* in bulk tank milk (BTM) and milk from primiparous (PP) cows sampled 4 times between September 2019 to August 2021. Negative = negative BTM and negative PP milk, PP positive = negative BTM and positive PP milk, BTM positive = positive BTM and positive or negative or missing PP milk. VG = Västra Götaland, ÖG = Östergötland.

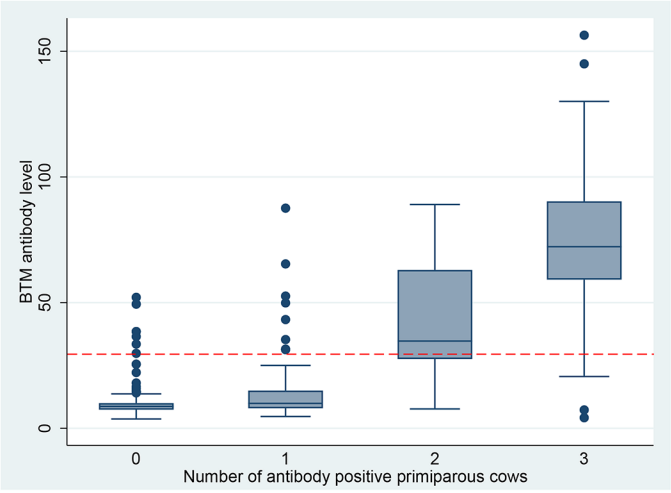

Figure 3. Bulk tank milk antibody level compared with the number of positive primiparous (PP) cows at all sampling occasions. The red dashed line represents the cut-off for the ELISA (S/P ≥ 30% = antibody positive). Herds with more than 3 sampled PP cows (n = 199) were transferred to one of the other categories by dividing the number of positive PP cows with the total number of PP cows sampled. Herds with less than 3 PP cows sampled (n = 13) were included in group 0, 1, or 2. The box is drawn from lower quartile (Q1) to the upper quartile (Q3) with a horizontal line drawn inside it to denote the median. The boundaries of the whiskers is based on the 1.5 IQR value from above Q3 and below Q1. The dots represents outliers outside the above range.

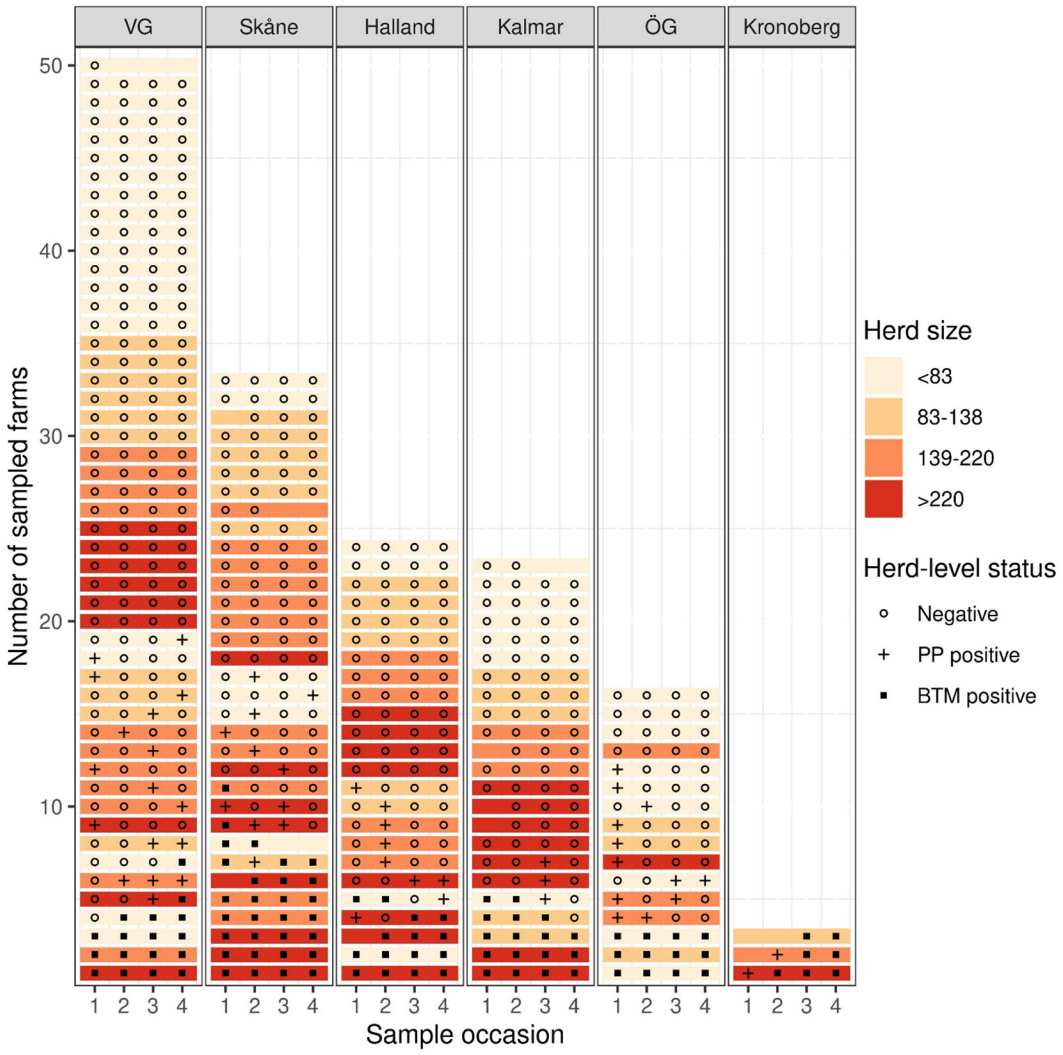

Figure 4. Herd-level results from analysis of antibodies to *Mycoplasma bovis* in bulk tank milk (BTM) and in individual milk from primiparous (PP) cows. Milk was sampled on 4 occasions: (1) autumn 2019, (2) spring 2020, (3) autumn 2020, and (4) spring/summer 2021. The symbols in the graph show the herd status at each sampling and the colors show the herd size. The herds are divided into columns by region, Västra Götaland (VG), Skåne, Halland, Kalmar, Östergötland (ÖG), and Kronoberg. Each horizontal line represents the time series for a single herd. If there is no symbol in the colored field, samples are missing.

1 PP cow positive, changes from negative to positive (on the next sampling) occurred 29 times (4 times in BTM and 25 times in PP). Changes from positive to negative at the next sampling occurred 34 times (5 times in BTM and 29 times in PP). Eleven herds went both from positive to negative and from negative to positive during the period of sampling. Most of the herds (n = 104, 70%) had the same status on all sampling occasions (22 positive and 82 negative). There were 67 herds with at least 1 positive sample. Out of these, all herds with negative BTM (n = 35) had both positive and negative PP samples. Most of the herds with positive BTM had also all PP samples positive (n = 24/32). Antibodies in either BTM or PP cows were detected in 63% (24/38) of the herds with more than 220 cows, 44% (16/36) in herds with 139–220 cows, 38% (14/37) in herds with 83 to 138 cows and 34% (13/38) in herds with less than 83 cows.

Risk Factors

In the initial screening of associations between *M. bovis* antibody status in PP cows and herd-level risk factors, 5 out of the 7 variables had a *P*-value of ≤0.20 and were further assessed in the model building procedure (Table 2). The variables region and breed had *P*-values above 0.20. In the final model, 3 variables remained significantly related to antibody status (Table 3). There were higher antibody levels in PP cows in larger herds, in

Table 2. Summary of the continuous variables showing number of individual milk samples from primiparous cows (n), median and interquartile range (IQR), for samples with no antibodies to *Mycoplasma bovis* (negative), and samples with such antibodies (positive) based on, in total, 2,448 samples from 143 herds at 4 different sampling occasions between September 2019 and August 2021[1]

	Negative			Positive			
Item	n	Median	IQR	n	Median	IQR	*P*-value[2]
Primiparous cow antibody level, S/P%	1,998	7.8	5.2–11.6	450	92.0	58.2–139.4	NA
BTM antibody level	1,997	8.7	7.5–10.2	441	66.4	49.9–82.1	<0.001
Herd size, cows	1,998	141	86–221	450	235	124–511	<0.001
No. of ingoing cattle	1,998	1	0–36	450	18	0–142	<0.001
Age of ingoing cattle, d	1,091	677	453–785	337	667	453–761	0.156
INHERDS[3]	1,998	1	0–2	450	2	0–4	0.088
Milk production[4]	1,866	11,060	10,359–11,740	383	11,321	10,476–12,129	0.886
BTM SCC[5]	1,865	244	196–288	393	241	193–273	0.974
Calf mortality 0–24 h[6] (%)	1,865	5.5	3.8–7.2	393	4.8	3.4–7.1	0.792
Early calf mortality (1–60 d)[6,7]	1,865	3	1–5	393	3	2–5	0.309
Late calf mortality (2–6 mo)[6,7]	1,865	1	0–2	393	1	0–2	0.142
Young stock mortality (6–15 mo)[6,7]	1,865	1	0–2	393	1	0–2	0.397
Cow mortality[6,7]	1,865	5	4–7	393	6	4–9	0.167
Culling of first parity cows in early (0–90 d) lactation[7]	1,865	3	1–6	393	4	2–7	0.310
Culling due to udder diseases[7]	1,865	8	5–11	393	8	5–11	0.930
Culling due to hoof and leg diseases[7]	1,865	3	2–5	393	4	2–5	0.202
Culling due to reproduction disorders[7]	1,865	8	5–10	393	8	5–11	0.178
Culling due to any reason including cow mortality[7]	1,865	35	31–41	393	38	34–42	0.143
All veterinary-treated diseases[7]	1,865	23	15–31	393	21	10–32	0.203
Veterinary-treated clinical mastitis[7]	1,865	10	6–14	393	8	4–10	0.223
Veterinary-treated hoof and leg diseases[7]	1,865	2	1–4	393	2	0–4	0.292
Calving interval,[8] mo	1,865	13	12–13	393	13	13–13	0.871
Age at first calving,[9] d	1,865	795	761–838	393	807	758–848	0.754
Heifers >17 mo not inseminated, %	1,865	15	6–27	393	17	7–34	0.463
Cows >70 d calving to first insemination,[10] %	1,865	17	11–24	393	16	13–20	0.953
Cows >120 d calving to final insemination,[11] %	1,865	6	5–8	393	7	5–8	0.479
Breed							
Swedish Red	278			202			Referent
Swedish Holstein	793			39			0.099
Mixed Red/Holstein	650			135			0.121
Other	173			20			0.604
Region							
Halland	373			48			Referent
Kalmar	295			47			0.941
Kronoberg	3			51			<0.001
Skåne	315			131			0.225
Västra Götaland	741			106			0.754
Östergötland	271			67			0.420

[1]Herd and sampling occasion were used as random effects. All variables have been calculated in 12-mo periods preceding sampling.

[2]*P*-value from univariable linear multilevel mixed regression with *M. bovis* antibody status in primiparous cows as outcome in the risk factor model. *P*-value from multilevel mixed-effects negative binomial models with health and production characteristics as outcome in the effect model.

[3]Number of herds where introduced cattle originate from in 12-mo period preceding sampling.

[4]Mean production per cow per 12 mo preceding sampling in kilograms of ECM.

[5]Bulk tank milk somatic cell count in 1,000 cells/mL, arithmetic mean of 12 monthly measurements.

[6]Mortality includes death and euthanasia per 12 mo preceding sampling.

[7]Cases per 100 animals at risk for 12 mo preceding sampling.

[8]Mean interval between latest calving and the calving before that, for all cows from second lactation giving birth during the 12-mo period preceding sampling.

[9]Mean age at first calving for heifers giving birth during the 12 mo preceding sampling.

[10]Number of cows, in the 12 mo preceding sampling, with an interval between calving and first insemination of >70 d divided by the mean number of cows with >70 d passed since calving (i.e., including cows calving within 70 d before the study period, not including cows calving within 70 d before the end of the study period).

[11]Number of cows, in the 12 mo preceding sampling, with an interval between calving and final insemination of >120 d divided by the mean number of cows with >120 d passed since calving (i.e., including cows calving within 120 d before the study period, not including cows calving within 120 d before the end of the study period).

herds that had introduced a higher number of cattle, and in herds with higher antibody levels in BTM. There were 2,248 observations from 143 herds and 4 different sampling occasions included in the analysis. The median age of the introduced animals was 674 d (IQR = 453–770). This variable was not included in the final model because

Table 3. Mixed linear regression model with herd and sampling occasion as random effects, regression coefficients with SE, and *P*-values evaluating herd-level variables associated with antibody status to *M. bovis* measured in milk from primiparous cows

Outcome	Predictor of interest	Coefficient	(SE)	*P*-value
M. bovis status primiparous cows	Herd size (cows)	0.001	(0.000)	0.003
	Introduction of cattle to the herd	0.001	(0.000)	0.006
(log-transformed)	BTM antibody level	0.025	(0.001)	<0.001

half of the herds had no introductions of cattle. When age was introduced in a similar model only including the herds with introductions of cattle, age of the introduced cattle was not a significant risk factor.

Effects on Health and Production

After the first univariable analysis there were 4 out of 20 variables (late calf mortality [2–6 mo], cow mortality, culling due to reproduction disorders, and culling due to any reason) with a *P*-value of ≤0.20 in the regression models (Table 2). These variables were further assessed in the model building procedure. Each of the 4 variables were tested in mixed negative binomial regression models correcting for the effect of breed, herd size, milk production, region, and introduction of cattle (yes or no). After evaluation of the models, there were no variables that remained significantly ($P \leq 0.05$) related to the herd-level antibody status in BTM and PP cows.

DISCUSSION

In the current study, sampling of BTM and milk from PP cows was repeated 4 times in 149 herds over 2 yr. The samples were analyzed for *M. bovis* antibodies with ID Screen ELISA. Together with *M. bovis* antibody status in the herds, data on introduction of cattle, herd size, health, and production in the herds were analyzed.

In this study, including individual samples from a few PP cows in addition to analyzing BTM alone increased the number of detected positive herds by 50% to 100%. The finding of positive PP cows and negative BTM suggests that few cows in the milking herd are seropositive. This could either be a result of a low within-herd transmission of *M. bovis* infection, or a recent introduction in the young cows. The risk of false positive test results contributing to this finding is low, around 1%, considering the high specificity (98.6%) of the ELISA (Andersson et al., 2019) and the prevalence of 18% detected among PP cows. In BTM positive herds, at least 30% of the lactating cows had antibodies in a study in Denmark (Petersen et al., 2016). In the few herds that showed positive BTM and negative PP cows, the sampling strategy failed to detect the antibody-positive cows, possibly older cows. Good biosecurity routines may have prevented the infection of young cows or *M. bovis* was no longer circulating in the herd, while historic antibodies were measured in BTM. In most herds with positive BTM, the PP samples were positive throughout the study period. In herds with negative BTM and positive PP cows, there was more variation in the PP results between samplings, probably due to a low within-herd seroprevalence and different cows sampled on each occasion (Petersen et al., 2016; Penterman et al., 2022). The BTM antibody levels in most of the positive herds did not decrease over a period of 2 yr. These findings support previous studies that BTM could be a good herd-level screening tool (Parker et al., 2017; Petersen et al., 2018; Salgadu et al., 2022).

Antibodies remain detectable in the herd after symptoms of clinical *M. bovis* disease have waned, but asymptomatic carrier animals may prevail, making the herd infection status difficult to assess (Maunsell et al., 2011; Penterman et al., 2022). Considering this, finding antibody-positive herds early is important in the work to prevent the spread of *M. bovis*. Antibody levels in PP cows have not been examined for *M. bovis* in other studies. The strategy to sample young cows was based on the assumption that they would reflect an active transmission of the infection in the herd better than older cows, since the duration of the antibody response (in the latter) may be quite long (Vähänikkilä et al., 2019). In previously *M. bovis*-free herds, PP cows might be the group first infected by *M. bovis* because they may have more contact with other herds, for example through external contractors rearing heifers from more than one dairy herd or by purchased animals being introduced into this age group. In this study, the median age of introduced animals was 674 d, approximately 22.7 mo, which seems to comply with the knowledge that pregnant heifers are the animals most often introduced to Swedish dairy herds. Therefore, we assumed that detecting antibody-positive PP cows would be a way to detect recent introduction of infection.

Introduction of cattle was correlated with increased antibody levels in PP cows. The introduction of cattle posed a significant risk factor, even though it also involved the movement of the farm's own cattle, which did not come into contact with cattle from other herds. It is well known that asymptomatic carrier animals play a big role in transmission of *M. bovis* (Maunsell et al., 2011). It has been shown that introduction of purchased animals

is a risk of getting *M. bovis* (Burnens et al., 1999) and this risk is decreased if herds only buy animals from controlled herds and only have other contacts (shared pasture, animal exhibitions) with *M. bovis* antibody negative herds (Dudek et al., 2020). There might, however, be a possibility that the infection is spreading to new herds through other pathways. The extent of transmission by fomites, clothes, humans, and semen is not fully understood, but these are possible risks of disease introduction (Haapala et al., 2018; Schibrowski et al., 2018). In the present study, larger herd size was a significant risk factor for having *M. bovis* antibodies. This was also previously shown in a study based on BTM samples from a national screening (Hurri et al., 2022) and in a study by Thomas et al. (1981). Larger herds and expanding herds have more contacts with other herds, both directly and indirectly, which entails a risk of introducing new pathogens (Fox et al., 2003; McAloon et al., 2022). The herds in our study had a median herd size of 150 cows (IQR = 87–247), which is higher than the mean herd size of 94 milking cows in Sweden (Swedish Board of Agriculture, 2019). This could have affected our results with a higher percentage of positive herds in the regions included in the present study (southern Sweden) compared with the national screening in 2019. Regions in the southern parts of Sweden were targeted in this study to find positive herds, making the results more comparable to other countries where *M. bovis* is endemic (Maunsell et al., 2011; Dudek et al., 2020). In this study about 17% of the herds were BTM antibody positive, compared with the national screening in 2019 where 8% (range 3%–20%) were positive in these regions (Hurri et al., 2022). This finding might reflect an increased prevalence over time, but it is also possible that farms with *M. bovis* infection were more prone to participate in the present study. However, the access to positive herds was beneficial for the study, because we wanted to investigate and monitor *M. bovis* over time. Therefore, we believe that the number of herds was satisfactory even though the participation rate was 14%, reflecting that willingness to participate in voluntary studies often is rather low.

Transmission of the disease between farms, indicated by new positive herds, was detected in all the regions during the study period. Geographic clustering of BTM positive herds was seen in the southeast part of region Skåne (Figure 3). Skåne was the region where *M. bovis* was first diagnosed in Sweden in 2011, and there were several positive herds near that farm in southeast Skåne, suggesting transmission by local contacts. Unfortunately, we could not investigate this further due to lack of data on distances and status of neighboring farms.

In the present study, data from the DHI database were used to explore the associations between herd-level antibody status to *M. bovis* in BTM and PP cows and herd health and production variables. In this analysis, we believed it was better to use antibody status as positive or negative on herd level instead of antibody levels, because the outcome variables were retrieved at herd level. Introduction of cattle to the herd may be a risk factor for the health and production by introducing other infections and therefore we also included this variable in the analysis of health and production. We could not find any associations between herd-level antibody status and health and production in this study. This is in contrast to an earlier study where *M. bovis* antibodies in BTM was associated with higher late calf mortality, young stock mortality, and a tendency of reduced fertility as measured by proportion of cows with more than 120 d from calving to final insemination (Hurri et al., 2022). Other studies have also shown health effects associated with having *M. bovis* antibodies, such as a lower milk production (Uhaa et al., 1990a; Timonen et al., 2017) and reproduction disorders (Uhaa et al., 1990b). The reason that we could not see any health effects in *M. bovis*-positive herds in this study could be due to unspecific data for calves, we had information on mortality but not on treatments. This kind of study might need a larger dataset to see differences between herds. Production and reproduction parameters are partly depending on the farmer's strategy and decisions, for example on when to inseminate and when to cull, information that was not available in this study.

CONCLUSIONS

In this study we show that analyzing antibodies in milk from PP cows, in addition to BTM, is a useful strategy to find herds infected with *M. bovis*. Higher antibody levels in PP cows were associated with larger herd size and a higher number of introduced cattle. Sampling PP cows can facilitate finding the infection in an early stage, thus enabling prevention of transmission both within and between herds.

NOTES

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ish National Data Service (https://doi.org/10.5878/c45h-ke16). The animal study was reviewed and approved by the regional ethics committee in Uppsala, approval number 5.8.18-02650/2019. Written informed consent was obtained from the farmers for participation in this study. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: BTM = bulk tank milk; INHERDS = number of herds that the introduced cattle originated from; IQR = interquartile range; ÖG = Östergötland; PP = primiparous; SH = Swedish Holstein; SLU = Swedish University of Agricultural Sciences; SR = Swedish Red; VG = Västra Götaland.

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