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Characterisation of the course of *Mycoplasma bovis* infection in naturally infected dairy herds

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ABSTRACT

Mycoplasma bovis causes bovine respiratory disease, mastitis, arthritis and otitis. The importance of *M. bovis* has escalated because of recent outbreaks and introductions into countries previously free of *M. bovis*. We characterized the course of *M. bovis* infection on 19 recently infected dairy farms over 24 months. Our objective was to identify diagnostic tools to assess the efficacy of control measures to assess low risk infection status on *M. bovis* infected farms. PCR assays and culture were used to detect *M. bovis*, and in-house and BioX ELISAs were used to follow antibody responses. Cows and young stock were sampled on four separate occasions, and clinical cases were sampled when they arose. On 17 farms, a few cases of clinical mastitis were detected, mostly within the first eight weeks after the index case. Antibodies detected by in-house ELISA persisted in the serum of cows at least for 1.5 years on all farms, regardless of the *M. bovis* infection status or signs of clinical disease or subclinical mastitis on the farm. Six out of 19 farms became low risk as the infection was resolved. Our results suggest that, for biosecurity purposes, regular monitoring should be conducted on herds by screening for *M. bovis* in samples from cows with clinical mastitis and calves with pneumonia, in conjunction with testing young stock by screening longitudinally collected nasal swabs for *M. bovis* and sequential serum samples for antibody against recombinant antigen.

1. Introduction

Mycoplasma bovis, an important pathogen of cattle, is most commonly associated with bovine respiratory disease (BRD), mastitis, arthritis, keratoconjuctivitis and otitis media (Nicholas and Ayling, 2003). The importance of *M. bovis* infection has escalated over the past decade because of the increasing number of outbreaks in major dairy producing countries, its recent introduction into countries that were previously free of *M. bovis*, including New Zealand and Finland, and the occurrence of an outbreak caused by novel strain (Pothmann et al., 2015; Biosecurity New Zealand, 2018; Haapala et al., 2018). *M. bovis* significantly affects animal welfare and production and causes significant economic loss in both beef and dairy cattle industries (Nicholas and Ayling, 2003). Efforts to develop efficacious vaccines against *M. bovis* have not been successful (Perez-Casal et al., 2017), with no commercial vaccines available throughout much of the world and only vaccines of questionable efficacy available in North America (Maunsell et al., 2011). Efforts to control the losses caused by *M. bovis* have led to increased use of antimicrobial drugs and recent reports suggest resistance to several antimicrobial drugs has increased in *M. bovis* (Ayling et al., 2014; Gautier-Bouchardon et al., 2014; Heuvelink et al., 2016). Therefore, detection of infected cattle, especially subclinically infected animals, is of paramount importance in prevention of introduction of the agent into naïve herds and in the prevention of outbreaks (Caswell and Archambault, 2007).

Several studies have examined the prevalence of infection with *M. bovis* using either PCR assays to detect the organism and/or serological assays, including enzyme-linked immunosorbent assays (ELISAs), to detect antibodies against it (Assie et al., 2009; Bednarek et al., 2012; Arede et al., 2016; Wawegama et al., 2016). At an individual animal level, asymptomatic carriage and intermittent shedding hamper reliable detection of the organism (Biddle et al., 2003; Hazelton et al., 2018).

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Infected animals may shed *M. bovis* for a few weeks to several months (Punyapornwithaya et al., 2010; Hazelton et al., 2018), but there is limited information about the circulation of *M. bovis* in an infected herd after natural infection and the antibody responses of animals in these infected herds.

In Finland *M. bovis* was detected in 2012, even though rigorous diagnostic testing had been performed to detect it in BRD research project of calf rearing units in 2001–2003 (Autio et al., 2007) and in clinical BRD samples since then. Furthermore, extensive screening for mastitis pathogens in individual milk samples from cases of clinical and subclinical mastitis has been conducted for decades in Finland, with approximately 140,000 samples were tested in 2018 (there are approximately 270,000 dairy cows in Finland). A multiplex PCR assay that included primers to detect *M. bovis* has been in use since early 2012. Between 2012 and 2018, 68 (0.8%) Finnish dairy herds were found to be infected with *M. bovis*.

The low prevalence of *M. bovis* and the low population density of cattle in Finland make it a suitable platform for studying the course of infection with *M. bovis* at the herd level. Cattle industries throughout the world need improved strategies and tools for certification of farms that have a low risk of infection with *M. bovis* for biosecurity purposes. In this study, we aimed to identify the best diagnostic tools to track the efficacy of control measures to achieve a low risk of infection with *M. bovis* on infected farms. To do this we followed the course of *M. bovis* infection in 19 recently infected dairy farms over 24 months using real time PCR and culture to detect the organism, and serology to detect the antibody response to *M. bovis,* in the herds over the study period. We used several sampling techniques on the farms, including collection of bulk tank milk samples, individual sampling of both cows and young stock by collection of quarter milk samples, nasal swabs, nasopharyngeal swabs and serum samples, and collection of samples during post-mortem examinations.

2. Materials and methods

2.1. Farms in the study

Animal Health ETT has maintained a voluntary *M. bovis* control program in Finland since 2013, and all the infected dairy herds were encouraged to participate in the control program and the research project "*Mycoplasma bovis* in dairy herds". The study included 19 infected dairy farms. Interviews and sampling were performed on each of these farms. Infection with *M. bovis* had not been detected previously on any of the farms, despite continuous mastitis pathogen testing (on 18 of the farms), including real time PCR for detection of *M. bovis*. On 17 farms the index cases were clinical mastitis confirmed by real time PCR, and on two farms the index cases were respiratory disease in calves. Table 1 shows the farm size, the management system, and the milk and post-mortem samples that were collected by farmers. The farms were advised to cull cows with mastitis caused by *M. bovis*, to avoid purchasing new animals, to house calves separately from the cows, and to follow appropriate hygiene measures.

2.2. Sampling

Veterinarians visited each of the farms four times, at approximately 6 month intervals, between 2014 and 2017. The dates of the visits are shown in Table 2. During each visit, the veterinarian collected deep nasopharyngeal (NP) swabs and nasal (NS) swabs from calves between 1 week and 9 months of age and serum samples from all age groups. A total of 5 NP and 10 to 20 NS swabs were collected at each visit, depending on the number of calves on the farm. The NS swab (Transystem, Copan, Brescia, Italy) was collected from each calf prior to collection of the NP swab. NP swabs were collected using 27 cm long guarded swabs (Medical Wire Equipment Ltd., Corsham, England) and soaked in mycoplasma D broth (Friis and Krogh, 1983). In herds with a sufficient number of young stock, 15 serum samples were collected from each age group of young stock (3–6, 6–9 and 9–12 months of age) and from cows, with a maximum 65 samples collected per herd. Farmers were advised to monitor cattle carefully for mastitis and other clinical signs and to submit quarter milk (QMS) samples from all cases of subclinical and clinical mastitis for real time PCR testing, and monthly bulk tank milk (BTM) samples for real time PCR and in-house mycoplasma immunogenic lipase A (MilA) ELISA (Wawegama et al., 2014). As part of the study, the farms could send other clinical samples, including calves with disease suspected to have been caused by *M. bovis* for post-mortem examination, culture and confirmation of *M. bovis* by real time PCR. Table 1 shows the total number of samples submitted by farmers over the study period.

2.3. Culture for M. bovis

NP swabs and clinical samples were cultured. Samples in mycoplasma D broth were sub-cultured onto F-medium plates and diluted tenfold in F broth (Bölske, 1988). The broths were incubated at 37 °C for 3–5 days, and growth and color change monitored every second day. All broth cultures were examined for the presence of *M. bovis* by real-time PCR, and suspected positive cultures were sub-cultured onto F-medium plates. Plates were incubated in 5% CO_2 at 37 °C for seven days, and inspected every second day under the microscope for mycoplasma colonies.

2.4. DNA extraction and M. bovis real time PCR

Nasal swabs and broth cultures were tested using a real time PCR assay targeting the *oppD* gene of *M. bovis* (CFX96 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, CA, USA), as described previously (Sachse et al., 2010), with the minor modifications described by Haapala et al. (2018).

QMS and BTM samples were analyzed by private laboratories using a commercial real time PCR assay for 16 mastitis pathogens, including *M. bovis* (Pathoproof® Complete 16-kit, Thermo Fisher Scientific, Finland), according to the manufacturer's instructions.

2.5. Detection of M. bovis-specific antibodies

Two ELISAs were used to detect *M. bovis*-specific antibodies, the Bio K260 (Bio-X Diagnostics, Jemelle, Belgium) commercial ELISA and an in-house IgG-detection MilA ELISA (Wawegama et al., 2014). This ELISA has an estimated animal-level sensitivity and specificity of 94.3% and 94.4%, respectively using a cut-off of 105 antibody units (AU) (Wawegama et al., 2016). The MilA ELISA was performed as described previously (Wawegama et al., 2014), with the concentration of *M. bovis* -specific antibodies in each sample calculated in antibody units (AU) by comparison with a series of standards on each plate using an ELISA analysis program (http://www.elisaanalysis.com), with a result of > 135 AU interpreted as positive (Petersen et al., 2018). The Bio K260 ELISA was performed according to the manufacturer's instructions.

BTM samples collected at or near the sampling visits were also tested using the in-house MilA ELISA essentially as described previously, but at a 1/20 dilution.

2.6. Grouping of the farms according to their infection status

To analyze the dynamics of herd *M. bovis*-specific antibody concentrations, we classified the study farms into six infection status groups, based on the detection of *M. bovis* at each visit (Table 3). We considered a visit positive if *M. bovis* was detected on the farm by real time-PCR or culture of NP or NS swabs, or clinical, post-mortem or mastitis samples, with the status at each visit classified as positive or negative. We designated the infection status S0 (n = 2) if *M. bovis* was detected only in the index cases, and not thereafter; S1 (n = 4) if *M.*

Table 1
Description of herds and number of milk and post-mortem samples collected by farmers.

Herd ID	No of cows	Barn type	Sample types, method, no of samples tested for *M. bovis*			
			QMS[1]	Bulk tank milk		Post mortem
			PCR	PCR	ELISA	Culture
A	47	tie-stall housing	64	16	4	
B	61	loose housing	252	16	4	2
C	183	loose housing	154	4	2	2
D	268	loose housing	500	2	2	
E	25	loose housing	27	16	4	
F	50	tie-stall housing	28	18	4	1
G	157	loose housing	487	19	3	
H	60	loose housing	194	17	4	3
I	100	loose housing	96	17	3	
J	61	loose housing	595	22	4	
K	29	tie-stall housing	51	12	4	1
L	41	tie-stall housing	51	6	3	
M	66	loose housing	112	18	4	1
N	158	loose housing	309	15	4	4
O	48	loose housing	94	13	4	1
P	18	tie-stall housing	12	11	4	
Q	28	tie-stall housing	30	18	4	
R	66	loose housing	36	12	4	1
S	127	loose housing	176	11	3	6

[1] QMS = quarter milk sample. Farmers were advised to monitor cattle for mastitis and other clinical signs thoroughly and to submit samples for testing for mastitis pathogens in all cases of subclinical and clinical mastitis by quarter milk sampling. Some farmers also submitted samples from clinically normal animals.

bovis was detected at the first sampling visit; S2 (n = 2) if *M. bovis* was detected at the first two sampling visits; S3 (n = 4) if *M. bovis* was detected at the first three sampling visits; S4 (n = 3) if *M. bovis* was detected at all four sampling visits; and Sx (n = 4) if *M. bovis* was detected at more than one visit, but not consistently throughout the period the herd was positive.

2.7. Statistical analysis

All statistical analyses were carried out using GraphPad Prism Version 7.02. The significance of the differences in the proportions of positive animals at each visit in each infection group were calculated using a one-way ANOVA and Tukey's multiple comparison test. $P < 0.05$ was considered significant. Median herd size was calculated, and Pearson's correlation coefficient was calculated to assess the association between herd size and the infection status of the farms, and the association between the bulk tank milk anti-MilA and serum anti-MilA antibody concentrations of the cows.

3. Results

3.1. Clinical manifestations and detection of M. bovis

On most of the farms the index case was clinical mastitis. There were only two farms on which no cases of mastitis caused by *M. bovis* were detected over the two year study period. On these farms the index cases were pneumonia in calves. On farms with *M. bovis* mastitis, only a few clinical mastitis cases were seen and these mainly (88%) occurred within the first eight weeks after the index case (Table 4). On most farms, cows with *M. bovis* mastitis were isolated, and slaughtered or culled as soon as possible after detection of infection. On one farm one cow was slaughtered later, at day 9 after detection, but it was kept separated from other animals throughout this period. Table 4 shows the results from testing farms for *M. bovis* at each visit and the infection status of each of the herds.

The farms were thoroughly examined for *M. bovis* over the 2 year study period (Tables 1,2,5). In total 3268 quarter milk samples were tested and only 51 cows had *M. bovis* mastitis. A total of 22 samples from 10 farms were submitted for post-mortem examination. *M. bovis* was isolated from 12 of these animals, from seven farms. PCR testing of 263 BTM samples, in total, yielded seven *M. bovis* positive samples, from five farms. All these positive samples, except for one, were collected within four weeks of the index mastitis case in the herd. On one farm (farm E), a single BTM sample was positive 5 months after the index case. After this positive finding, quarter milk samples from cows on farm E with elevated milk somatic cell counts were tested for *M. bovis*, and all were negative. Two cows with subclinical mastitis were dried off in the meantime without sampling. However, NS and NP swabs from calves on farm E were positive.

The median size of the herds was 61 cows and there was a positive, moderate correlation between herd size and the infection status of the farm (Pearson r = 0.6). On infection status S0 farms (n = 2) *M. bovis* was only detected in the index case and was not isolated at any other time throughout the study period. On four farms (infection status S1) *M. bovis* was detected only in the first half of the first year and on two farms (infection status S2) it was detected throughout the first year. On 11 farms (infection status S3, S4 or Sx) *M. bovis* was detected after the first year, and the majority of these farms had large herds in loose housing barns.

3.2. Serum antibodies against M. bovis

A total of 3317 serum samples were tested using two different ELISAs, using a cut-off values 135 AU for the MilA ELISA and an ODC of 37% for the BioX ELISA (Table 5). The MilA ELISA detected more positive samples than the BioX ELISA. We analyzed the *M. bovis* specific antibody profiles for each infection status group using both ELISAs (Figs. 1 and 2). The proportions of MilA ELISA positive young stock had patterns similar to those detected by culture or PCR for *M. bovis*. In contrast, no such patterns were obtained with the BioX ELISA. There was a significant decrease in the proportion of young stock that were positive in the MilA ELISA after the first sampling visit ($P < 0.05$) on S0 and S1 farms (Fig. 1 a, b). Similarly, there was a significant decrease in the proportion of young stock that were positive in the MilA ELISA after the second visit on S2 farms ($P < 0.05$, Fig. 1 c). In contrast, on S3, S4 and Sx farms the proportion of young stock that were positive in the MilA ELISA remained as high as 80% at most time points, without any significant decrease in the proportion that were positive over the

Table 2
Infection status at each visit to each herd based on detection of *M. bovis*.

			Visit 1					Visit 2					Visit 3					Visit 4				
						No. positive/ total[2]					No. positive/ total					No. positive/total					No. positive/ total	
Herd	Index case	Infection status[1]	Date of visit	Infection status	No. infected calves	NS	NP	Date of visit	Infection status	No. infected calves	NS	NP	Date of visit	Infection status	No. infected calves	NS	NP	Date of visit	Infection status	No. infected calves	NS	NP
A	29th Apr 2014	S3	10th Nov 2014	+	2/19	2/19	2/5	4th May 2015	+	3/15	1/15	2/6	10th Nov 2015	+	1/21	1/21	0/5	23rd May 2016	–	0/19	0/19	0/5
B	21st Apr 2015	S2	19th May 2015	+	11/19	10/19	4/5	24th Nov 2015	+	3/23	3/23	0/5	17th May 2016	–	0/23	0/23	0/5	21st Nov 2016	–	0/19	0/19	0/5
C	14th Jan 2016	S4	25th Jan 2016	+	11/20	10/20	4/5	24th Aug 2016	+	12/20	12/20	3/5	15th Feb 2017	+	4/19	3/19	1/5	29th Aug 2017	+	1/20	1/20	0/5
D	12th Aug 2013	S4	8th Oct 2013	+	24/50	24/50	ND	10th Dec 2014	+	10/20	10/20	2/5	8th Sept 2015	+	2/20	2/20	2/10	22nd Mar 2016	+	8/21	7/21	1/5
E	10th Mar 2015	S3	30th Mar 2015	+	9/15	9/15	3/5	19th Oct 2015	+	1/9	0/9	1/5	25th Apr 2016	+	6/12	4/12	5/5	24th Oct 2016	–	0/13	0/13	0/5
F	12th May 2015	S2	27th May 2015	+	4/11	4/11	1/5	1st Dec 2015	+	3/16	2/16	2/5	18th May 2016	–	0/13	0/13	0/5	23rd Nov 2016	–	0/13	0/13	0/5
G	15th Jan 2016	Sx	21st Feb 2016	+	10/20	10/20	3/5	1st Sept 2016	–	0/20	0/20	0/5	28th Feb 2017	+	3/19	3/19	0/5	22nd Aug 2017	–	0/20	0/20	0/5
H	5th Nov 2015	Sx	14th Dec 2015	+	4/15	3/15	3/5	8th June 2016	+	9/19	9/19	3/5	12th Dec 2016	–	0/14	0/14	0/5	20th June 2017	+	1/19	1/19	0/5
I	23rd Dec 2015	S3	3rd Feb 2016	+	3/19	2/19	1/5	9th Aug 2016	+	6/18	6/18	4/5	7th Feb 2017	+	1/20	1/20	0/5	23rd Aug 2017	–	0/20	0/20	0/5
J	11th Dec 2015	S1	11th Jan 2016	+	15/20	14/20	4/5	12th July 2016	–	0/18	0/18	0/5	3rd Jan 2017	–	0/27	0/27	0/5	4thJuly 2017	–	0/20	0/20	0/5
K	17th Jul 2014	S0	11th Nov 2014	–	0/6	0/6	0/5	11th May 2015	–	0/8	0/8	0/5	24th Nov 2015	–	0/9	0/9	0/5	24th May 2016	–	0/7	0/7	0/5
L	24th May 2014	Sx	10th June 2014	+	7/21	7/21	6/6	27th Nov 2014	–	0/20	0/20	0/5	25th May 2015	+	9/20	9/20	3/5	16th Nov 2015	+	7/14	7/14	0/5
M	29th Aug 2014	S0	12th Nov 2014	–	0/9	0/9	0/5	11th May 2015	–	0/15	0/15	0/6	25th Nov 2015	–	0/18	0/18	0/5	24th May 2016	–	0/13	0/13	0/5
N	18th Mar 2015	S3	15th Apr 2015	+	11/23	9/23	2/5	10th Nov 2015	+	16/25	14/25	4/5	16th May 2016	+	11/20	8/20	4/5	22nd Nov 2016	–	0/20	0/20	0/5
O	31st Mar 2015	S1	28th Apr 2015	+	4/16	4/16	0/6	3rd Nov 2015	–	0/19	0/19	0/5	26th Apr 2016	–	0/19	0/19	0/5	21st Nov 2016	–	0/15	0/15	0/5

(*continued on next page*)

Table 2 (*continued*)

Herd	Index case	Infection status[1]	Visit 1					Visit 2					Visit 3					Visit 4				
			Date of visit	Infection status	No. infected calves	No. positive/ total[2]		Date of visit	Infection status	No. infected calves	No. positive/ total		Date of visit	Infection status	No. infected calves	No. positive/total		Date of visit	Infection status	No. infected calves	No. positive/ total	
						NS	NP				NS	NP				NS	NP				NS	NP
P	29th Oct 2015	S1	25th Nov 2015	+	1/6	1/6	0/5	31st May 2016	–	0/9	0/9	0/4	29th Nov 2016	–	0/8	0/8	0/5	16th May 2017	–	0/7	0/7	0/5
Q	12th Aug 2015	S1	9th Sept 2015	+	2/19	2/19	0/5	16th Mar 2016	–	0/15	0/15	0/5	20th Sept 2016	–	0/11	0/11	0/5	14th Mar 2017	–	0/12	0/12	0/5
R	1st Sept 2015	Sx	20th Oct 2015	+	5/20	5/20	3/5	18th Apr 2016	–	0/21	0/21	0/5	17th Oct 2016	–	0/18	0/18	0/5	4th Apr 2017	+	13/21	10/ 21	5/5
S	25th Jan 2016	S4	16th Feb 2016	+	8/11	8/11	5/5	16th Aug 2016	+	7/20	6/20	4/5	13th Feb 2017	+	5/20	3/20	3/5	8th Aug 2017	+	1/15	0/15	1/4

[1] S0 = Mb detected in index case. S1 = Mb detected in index case and at first visit. S2 = Mb detected in index case and at first and second visits. S3 = Mb detected in index case and at first, second and third visits. S4 = Mb detected in index case and at all subsequent visits Sx = Mb detected intermittently. [2] No. of samples. NS = nasal swab. NP = nasopharyngeal swab. ND = not done.

duration of the study (Fig. 1. d, e, f). However, 80 to 100% of cows were MilA ELISA positive throughout the duration of the study, regardless of the infection status of the farm. There were a few exceptions at a few time points, but no significant difference between the time points. On S0 farms, there was a significant decrease in the proportion of positive cows after the first visit, but the proportion had increased again by the third visit. On S4 and Sx farms, all the cows tested were positive in the MilA ELISA at the start of the project, and approximately 80% were positive at each time point thereafter (Fig. 1 e, f). No patterns were detectable in the BioX ELISA results from the different infection status groups (Fig. 2), possibly because the overall number of animals that were positive in the BioX ELISA was much lower than in the MilA ELISA. However, there was a significant decrease in the proportions of young stock and cows on S4 farms that were positive in the BioX ELISA after the first sampling (Fig. 2 e).

3.3. Anti-*M. bovis* antibodies in bulk tank milk

The concentrations of anti-*M. bovis* antibodies in the bulk tank milk samples (n = 68) were compared to the mean concentration of anti-*M. bovis* antibodies in the serum samples collected from the cows in the herd at the same visit over all four visits. There was a positive correlation between the anti-*M. bovis* antibody concentrations in the bulk tank milk samples and those in sera of the cows (r = 0.45), as measured using the MilA ELISA (Fig. 3).

4. Discussion

This is the first study to examine the circulation of *M. bovis* and the antibody responses against it in naturally infected dairy farms over a period of two years. We examined 19 farms for the presence of *M. bovis* using repeated individual mastitis milk and BTM sampling, and sampling of young stock by nasal and deep nasopharyngeal swabbing and collection of serum samples from young stock and cows on four occasions over two years.

In the majority (88%) of the farms, clinical *M. bovis* mastitis was only detected over a short period, and the number of mastitis cases associated with *M. bovis* remained low. A short period of *M. bovis* mastitis could result in the early detection of mastitis and in a rapid response to remove the animals from the farm. Previous studies have found that the length of a mastitis outbreak can vary from two months to several years (Bayoumi et al., 1988; Mackie et al., 2000; Fox et al., 2003; Punyapornwithaya et al., 2012). On six farms (J, K, M, O, P and Q), *M. bovis* was not detected on three or more consecutive visits, with all milk samples (both QMS and BTM) negative for *M. bovis* over that time. Clinical disease was resolved on these farms with herds having cleared *M. bovis* thus becoming low risk. These farms seemed to have not only resolved the clinical disease, but also seemed to have cleared the herd of *M. bovis* and thus achieved low risk status. Previous studies have demonstrated that herd size is a risk factor for *M. bovis* mastitis (Thomas et al., 1981; Fox et al., 2003; McCluskey et al., 2003; Lysnyansky et al., 2016). All the herds in our study that appeared to have been cleared of *M. bovis* had fewer than 70 cows, and the majority of the farms in which infection persisted had larger herds though none exceeding 268. However, it has been shown that herds with over 500 cows are more vulnerable to mycoplasma mastitis than smaller herds (Nicholas et al., 2016). Several factors are associated with minimizing clinical disease and resolving outbreaks. Most of these are essentially management practices that minimize the risk of transmission, including separation of clinically affected animals from the herd, removal/culling of other infected animals, milking the hospital herd last and pasteurizing milk before feeding it to calves (Fox et al., 2003; Hazelton et al., 2018). To develop effective control strategies and *M. bovis* eradication programs, we need more investigations to characterize the most important farm management practices, to determine optimal biosecurity measures, and to determine the pathogen and host factors associated

Table 3
Classification of the infection status of herds based of detection on *M. bovis* at repeated sampling visits.

Infection status	Visit 1	Visit 2	Visit 3	Visit 4	Herd
S0[1]	–	–	–	–	K, M
S1	+	–	–	–	J, O, P, Q
S2	+	+	–	–	B, F
S3	+	+	+	–	A, E, I, N
S4	+	+	+	+	C, D, S
Sx	+	–	+	–	G
Sx	+	+	–	+	H
Sx	+	–	+	+	L
Sx	+	–	–	+	R

[1] S1 = Mb detected in index case and at first visit. S2 = Mb detected in index case and at first and second visits. S3 = Mb detected in index case and at first, second and third visits. S4 = Mb detected in index case and at all subsequent visits Sx = Mb detected intermittently.

with clearance of *M. bovis* from a herd.

Studies on *M. bovis* in dairy herds have focused on herds experiencing outbreaks of clinical mastitis (Hazelton et al., 2018; Petersen et al., 2018). In our study, the majority of the farms were infected, but had very few clinical cases, and could be therefore be defined as infected farms that were not experiencing an outbreak. On two farms (O and R), there were no *M. bovis* mastitis cases, even though *M. bovis* was circulating (Farm O, with an S1 infection status and Farm R with an Sx status). The cows had detectable serum antibodies against *M. bovis*, showing that they had been exposed to the pathogen but had not developed any signs of *M. bovis* mastitis. Similarly on two farms (A and E), *M. bovis* was circulating for over one year, but no *M. bovis* mastitis cases were seen after the index cases. Thus, continuous QMS testing of subclinical and clinical mastitis for *M. bovis* alone is not sufficient to detect the presence of *M. bovis* in a herd, even though it is crucial for detection of cases of clinical mastitis.

Routine identification of herds with *M. bovis* mastitis has typically been performed using BTM culture or PCR. The collection of BTM samples at intervals of 3 or 4 days is an effective way to screen for mycoplasma mastitis in dairy herds (Wilson et al., 2009). Screening for *M. bovis* in BTM has also been recommended as a biosecurity measure to reduce the risk of introduction of *M. bovis* into a naïve herd (Parker et al., 2017). In our study, PCR positive BTM samples were detected in only five (26%) of the herds, and only shortly after the initial mastitis cases, although *M. bovis* was still circulating in many of the herds for an extended time after this. Thus, a negative PCR test result on BTM is not an accurate method for determining the absence of *M. bovis* in a herd, and, therefore, is not suitable as the sole test for biosecurity screening, even though it is suitable for detecting *M. bovis* mastitis.

Table 5
Results from testing individual serum samples for antibodies against *M. bovis* using the BioX and MilA ELISAs (number positive/total number of samples).

	Visit 1		Visit 2		Visit 3		Visit 4	
Herd	BioX	MilA	BioX	MilA	BioX	MilA	BioX	MilA
A	2/36	33/36	2/37	20/37	2/38	25/38	1/37	7/37
B	10/54	46/54	5/57	54/57	5/61	33/61	1/47	21/47
C	27/61	54/61	8/61	53/61	5/60	60/60	19/61	57/61
D	15/30	29/30	9/59	48/59	7/60	53/60	6/60	60/60
E	7/32	31/32	5/30	24/30	3/28	26/28	3/30	22/30
F	17/30	24/30	4/26	22/26	2/30	23/30	0/31	17/31
G	1/61	36/61	5/60	40/60	1/55	54/55	10/47	42/47
H	23/38	38/38	7/30	27/30	2/39	31/39	1/31	14/31
I	8/53	45/53	17/44	40/44	3/41	36/41	19/53	50/53
J	18/36	30/36	5/41	28/41	3/34	19/34	2/36	10/36
K	0/25	22/25	2/22	11/22	2/26	16/26	2/26	9/26
L	17/29	29/29	0/44	30/44	3/45	42/45	7/38	33/38
M	2/35	34/35	3/34	16/34	4/43	16/43	0/40	16/40
N	26/65	54/65	18/64	53/64	4/60	60/60	2/60	41/60
O	4/31	27/31	2/32	13/32	1/35	28/35	1/35	20/35
P	2/19	16/19	3/21	15/21	2/24	17/24	1/21	11/21
Q	7/32	28/32	5/31	24/31	3/33	13/33	1/31	15/31
R	8/42	36/42	3/43	24/43	3/32	14/32	8/46	38/46
S	11/17	15/17	14/35	33/35	7/30	29/30	2/22	22/22

Our study has shown that on all farms the majority of cows were seropositive in the MilA ELISA, with antibodies against *M. bovis* detected for at least for 1.5 years, regardless of the apparent ongoing presence or absence of *M. bovis* in the farm. Most previous studies have used other ELISAs and concluded that serum antibodies do not remain elevated for a prolonged period. Recently, Petersen et al. (2018) studied *M. bovis* infected cows using the BioX ELISA and showed that serum antibody levels generally declined within 2 months after the onset of clinical disease. They concluded that serology is unlikely to be useful for individual diagnosis of disease associated with *M. bovis* in dairy cows. In our study seropositive cows were detected using the BioX ELISA at most of the time points. However, after the second visit 20% or fewer of the cows or young stock were seropositive, regardless of the infection status of the farm. Therefore, the BioX ELISA results did not reflect the circulation of *M. bovis* in different infection status groups. However, these results were not unexpected, as the BioX ELISA has

Table 4
Detection of *M. bovis* infection and *M. bovis* mastitis during the study period.

Herd	Index case	Infection status	No. *M. bovis* mastitis cases	Week 1	Weeks 2 - 8	Weeks 8 - 26	Weeks 27 - 52	After 53 weeks
A	Mastitis	S3	1	1				
B	Mastitis	S2	2	1	1			
C	Mastitis	S4	8	3	4	1		
D	Mastitis	S4	6	1	3			2
E	Mastitis	S3	1	1				
F	Mastitis	S2	1	1				
G	Mastitis	Sx	1	1				
H	Mastitis	Sx	4	1	3			
I	Mastitis	S3	7	1	4	2		
J	Mastitis	S1	3	1	2			
K	Mastitis	S0	1	1				
L	Mastitis	Sx	1	1				
M	Mastitis	S0	1	1				
N	Mastitis	S3	7	2	4		1	
O	Calf pneumonia	S1	0					
P	Mastitis	S1	2	1	1			
Q	Mastitis	S1	1	1				
R	Calf pneumonia	Sx	0					
S	Mastitis	S4	4	1	3			
		Total (%)	51	20 (39 %)	25 (49 %)	3 (6 %)	1 (2 %)	2 (4 %)

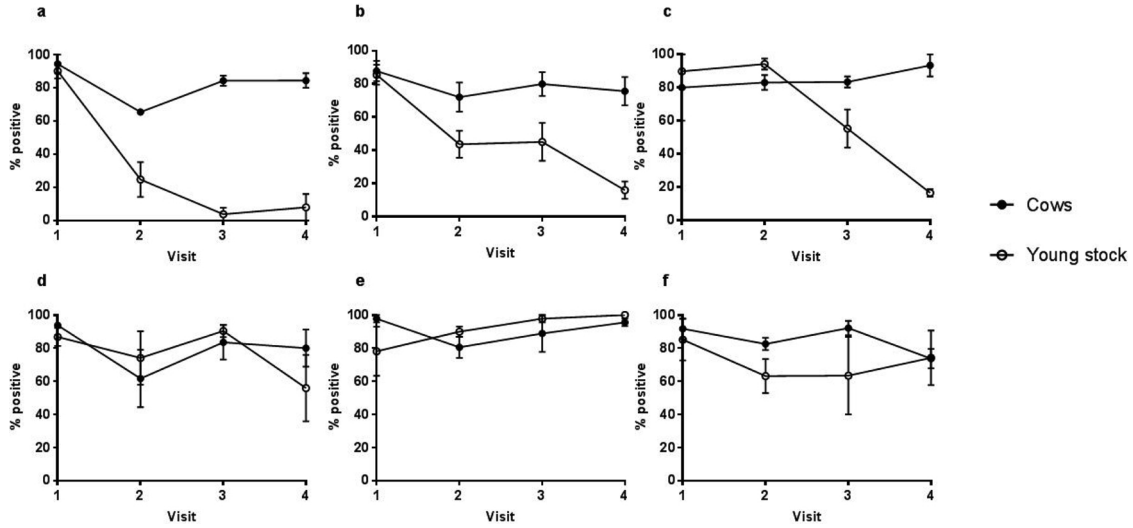

Fig. 1. Proportions of animals seropositive over time in herds with different statuses of infection using the MilA ELISA. a) infection status S0, b) infection status S1, c) infection status S2, d) infection status S3, e) infection status S4, f) infection status Sx.

been found to have a much lower sensitivity than the MilA ELISA (Wawegama et al., 2016; Petersen et al., 2018).

Serum antibodies against *M. bovis* were detectable in the majority of the cows using the MilA ELISA throughout the study period. Strikingly, there was a clear decline in serum anti-MilA antibody concentrations in young stock on the farms on which infection was under control, and on which *M. bovis* was not detected in the second year after the index case. In contrast, on S3, S4 and Sx farms, where *M. bovis* circulated for more than 1.5 years, 60 to 80% of young stock remained seropositive. Serological diagnosis alone indicates past exposure rather than current infection (Nicholas and Ayling, 2003). Nevertheless, our study suggests that sequential testing of different age groups for antibodies against *M. bovis* can be used to assess the *M. bovis* infection status of a herd and the trend towards clearance of infection from a herd to achieve a low risk *M. bovis* status. There was also a correlation between the serum antibody concentrations of the cows in a herd and the BTM antibody concentrations. This suggests that BTM antibodies may reflect not only antibodies produced in the udder but also those in the serum, or alternatively that intramammary infections with *M. bovis* can induce a detectable systemic immune response. More investigation of appropriate cut-off values and robust interpretations for use of the MilA ELISA for testing BTM would result in a powerful diagnostic tool for following the infection status of herds for eradication or control of *M. bovis* in dairy herds.

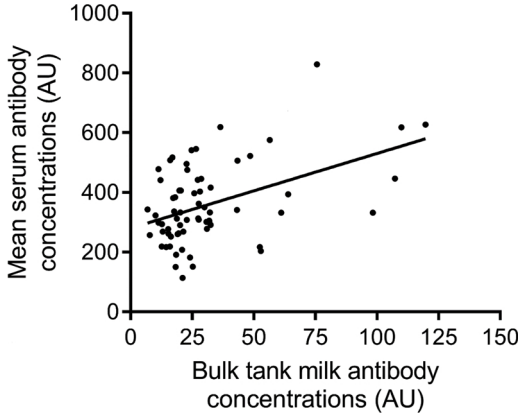

Fig. 3. Correlation between mean serum antibody concentrations (AU) of the cows in each herd and the bulk tank milk antibody concentration at the same collection time points using the MilA ELISA. Line represents r = 0.45.

Previous studies have shown that during an initial outbreak of *M.*

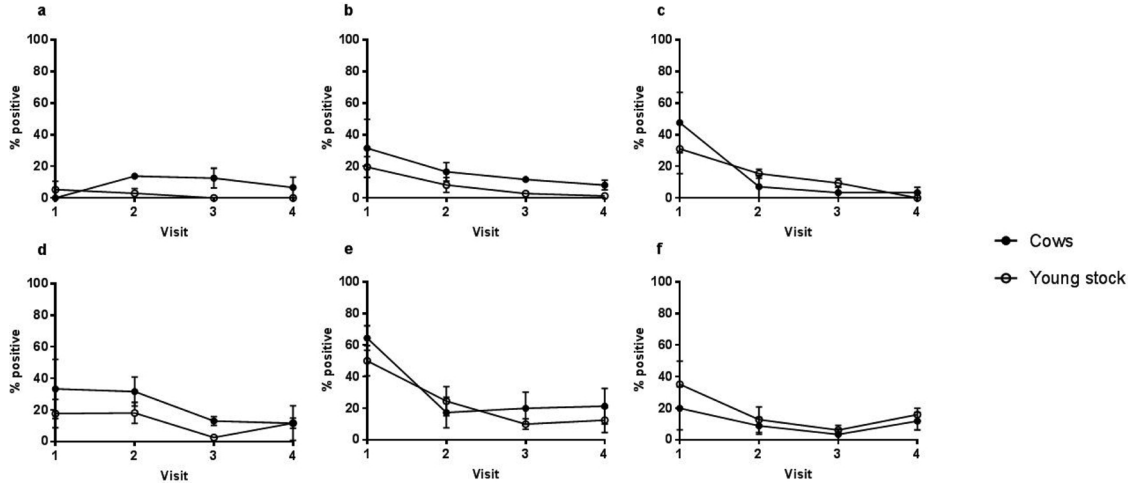

Fig. 2. Proportions of animals seropositive over time in herds with different statuses of infection using the BioX ELISA. a) infection status S0, b) infection status S1, c) infection status S2, d) infection status S3, e) infection status S4, f) infection status Sx.

bovis mastitis, colonization of distant sites in the body may be common in cows, but that this decreases rapidly (Punyapornwithaya et al., 2010; Hazelton et al., 2018). Thus, timing of sampling in relation to a recent outbreak appears to be of considerable importance and sampling of body sites by nose, eye, or vaginal swabs of cows is ineffective in evaluating the presence of *M. bovis* in the herd. In our study, we did not directly test for the colonization of cows with *M. bovis*, but the high levels of antibodies against *M. bovis* in cows on farms without ongoing disease appears likely to reflect frequent asymptomatic infection with *M. bovis*. Moreover, in young stock the patterns of seropositivity appeared to reflect the presence of the pathogen.

Calves are believed to shed *M. bovis* in respiratory secretions and thus to transmit infection through continued close contact (Nicholas, 2011). In our study, the prevalence of detection of *M. bovis* in calves ranged from 0 to 75% on different visits. On several farms, *M. bovis* was detected in nasal and nasopharyngeal swabs from calves when there had not been any cases of clinical *M. bovis* mastitis in cows in the previous months. It has been shown that the within-herd prevalence of detection of *M. bovis* in nasal swabs from calves is generally high (up to 100%) in herds with a history of an outbreak, and low in herds that have not experienced an outbreak (Maunsell and Donovan, 2009). These results, combined with our findings, emphasize that effective biosecurity screening requires repeated monitoring of calves to ensure that the presence of *M. bovis* on a farm is detected.

For the biosecurity purposes, it is critically important to prevent introduction of *M. bovis* into a herd by purchasing animals from farms shown to be free of infection. Shedding of *M. bovis* has shown to be intermittent (Caswell and Archambault, 2007; Maunsell et al., 2011). Therefore, a single test of an individual animal is not reliable, and the infection status of the herd needs to be determined by sequential testing using serology and PCR. There is no gold standard for sampling and testing asymptomatic animals. In this study, we could not interpret the Bio-X ELISA results with respect to the infection status of the herds, as similar levels of seropositivity were seen in herds whether there was ongoing circulation of *M. bovis* or not. Similarly, although the MilA ELISA results appeared to mirror the infection status of the young stock, the proportion of cows that were seropositive cows was high in all infection status groups and thus these animals may not be the best sentinels for biosecurity screening. In conclusion, our study suggests that regular monitoring for *M. bovis* in samples from cows with mastitis and calves with pneumonia, combined with longitudinal sampling of young stock by nasal swabbing for PCR and for serum for testing in the MilA ELISA, and potentially testing bulk tank milk for antibodies against *M. bovis* using the MilA ELISA, provide suitable diagnostic tools for biosecurity screening and control programs.

5. Conclusions

If control of *M. bovis* is to be improved, it is critically importance to prevent the introduction of *M. bovis* into herds by purchasing animals from farms that are free of infection. Testing of individual animals or herds on a single occasion is not reliable, so the infection status of the herd needs to be based on sequential testing of herds. Our results suggest that optimal assurance of the infection status of herds can only be achieved by regular monitoring for *M. bovis* in samples from clinical cases of mastitis and calf pneumonia, combined with longitudinal collection from young stock of nasal swabs for detection of *M. bovis* by PCR testing and sera for detection of antibodies against *M. bovis* using the MilA ELISA.

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