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Dynamics of *Mycoplasma bovis* in Dutch dairy herds during acute clinical outbreaks

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ABSTRACT

Mycoplasma bovis (*M. bovis*) can cause serious illness in cattle, presenting as arthritis and mastitis in dairy cows and pneumonia, arthritis and otitis media in calves. This study aimed to provide insight into the dynamics of *M. bovis* within dairy herds, experiencing an acute outbreak in dairy cows. Twenty farms were followed with laboratory testing of suspected dairy cows. Each outbreak farm was sampled five times, at 2–3 week intervals, sampling blood and milk and conjunctival fluid from clinically suspected dairy cows and healthy animals from three different age groups: dairy cows, young stock (7–24 months) and calves (1–6 months). Additionally, bulk tank milk was sampled every visit and environmental samples were taken on the first and last visits. The presence of *M. bovis* was tested by evaluating antibody titres in blood, bacterial DNA in conjunctival fluid and environmental samples and viable bacteria in milk samples. All data were analysed using logistic regression models, corrected for repeated sampling and within-herd correlation.

Sixty percent (12/20) of the herds showed a combination of arthritis and mastitis, while other herds experienced only clinically mastitis (3/20) or arthritis (5/20). From the time an outbreak was confirmed, *M. bovis* infection was not only present in dairy cows, but also in young stock and calves (80% of the farms). Laboratory tests also confirmed the presence of *M. bovis* in healthy animals. The *M. bovis* PCR levels of calves and young stock were highly correlated at all visits ($r_{total} = 0.81$, $P < 0.01$). Furthermore, *M. bovis* was present in the environment of the animals. At the end of the 3-month study period, none of the 20 clinical outbreak farms were *M. bovis*-'negative', based on laboratory testing, although hardly any clinical cases were observed at that time.

1. Introduction

Mycoplasma bovis (*M. bovis*) can cause serious illness in cattle, including arthritis, mastitis, pneumonia and reproductive disorders in dairy cows (Maunsell et al., 2011; Haapala et al., 2018; Hazelton et al., 2018) and pneumonia, arthritis and otitis in calves (Maunsell and Donovan, 2009). *Mycoplasma* spp. can be present in the microbiome of the upper respiratory tract (Lima et al., 2016) and may be present in semen of bulls and in vaginas of dairy cows (Haapala et al., 2018; Hazelton et al., 2020). Not all infected animals show clinical signs; asymptomatic carriers may shed the pathogen for long time (Nicholas et al., 2016; Punyapornwithaya et al., 2010; Wilson et al., 2007). *M. bovis* bacteria can be detected with microbiological culture (e.g., milk) and polymerase chain reaction (PCR) methods (e.g. conjunctival swabs). Indirect enzyme-linked immunosorbent assays (ELISAs) demonstrate the presence of antibodies directed against *M. bovis* in, for example, serum and milk samples. These three diagnostic methods are recommended for complementary use because all exhibit limitations (Parker et al., 2018). Factors such as the presence of sub-clinical infections and intermittent shedding also complicate the diagnosis. Therefore repeated sampling of individuals is recommended to increase the likelihood of *M. bovis* detection in both clinically and sub-clinically infected animals (Hazelton et al., 2018; Parker et al., 2018; Petersen et al., 2018).

The objective of this study was to provide new insights into the dynamics of *M. bovis* transmission between and within age groups in dairy herds experiencing an acute clinical outbreak in dairy cows.

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2. Materials and methods

Between February 2016 and April 2017, GD Animal Health (GD) performed a longitudinal study in which a cohort of 20 Dutch dairy herds was closely followed, that experienced an acute clinical *M. bovis* outbreak in dairy cows. The study was performed according to the Directive 2010/63/EU on the protection of animals used for scientific purposes, and approval was granted by the Dutch Central Authority for Scientific Procedures on Animals (license number 2015300) and the Animal Welfare Body of GD.

2.1. Selection of farms

Practitioners were asked to report dairy herds with several dairy cows with *M. bovis*-associated clinical signs of mastitis or arthritis within a 2-week period, confirmed by a confirmative laboratory test result for *M. bovis* (ELISA, PCR or culture from at least two cows). Only farms larger than 50 dairy cows were included which had no *M. bovis* clinically suspected animals in the preceding 12 months.

2.2. Study design

The outbreak farms were visited five times; within a week after confirmation of the outbreak (v0) and after 2 (v2), 5 (v5), 8 (v8) and 11 (v11) weeks. Dairy cows with clinical signs of mastitis or arthritis ('clinically suspected') were enroled in the study, with a maximum of five cows per visit. Clinically healthy animals from three age groups (hereafter: 'healthy animals') were randomly selected prior to the first visit, using a selection list with a random number generated for each animal in the farm ('runiform' in Stata14/SE; StataCorp, Stata Statistical Software, 2015, Release 14). Cattle with the lowest selection number were selected for inclusion in the study. Groups included a cohort of a maximum of 13 dairy cows older than 24 months of age, 13 young stock between 7 and 24 months and 10 calves between 1 and 6 months and were sampled during each visit. When an animal was lost to follow-up due to culling, drying off or calving, a healthy animal that was next on the selection list or a new clinically suspected animal, was included during the following visit.

Samples were collected by trained veterinarians according to standard operating procedures. Serum blood samples were collected from the coccygeal vein. Conjunctival fluid samples from the medial eye corners of both eyes were collected with a dry sterile flocculated swab (Eswab, Copan, Italy) and directly stored in the standard transportation medium. Environmental samples were collected and stored only during v0 and v11,with the same Eswab system. Composite milk samples from four quarters and BTM were collected in sterile 10-mL and 50-mL tubes, respectively. All samples were cold stored and immediately sent to the laboratory for diagnostic analyses.

2.3. Laboratory processing of samples

Serum samples were analysed with the commercially available indirect ELISA kit for *M. bovis* antibody detection (BioX K260 Diagnostics, Rochefort, Belgium) conform the manufacturer's instructions. Based on these instructions, results were reported in six categories: – (no antibodies detected) and 1 + to 5 + (increasing amounts of antibodies detected), whereby 1 + and 2 + were considered a low positive response and 3 + or higher as a high positive response.

M. bovis DNA was detected by PCR in conjunctival fluid using an in-house competitive allele-specific, real time PCR. Briefly, DNA was isolated using an ABI MagMAX™ Total Nucleic Acid Isolation kit on a MagMax Express machine. The *M. bovis* specific primers were Fw primer 5′-GAA GGT GAC CAA GTT CAT GCT GGC AAA CTT ACC TAT CGG TGA C-3′ and Rev primer 5′-AGG CAA AGT CAT TTC TAG GTG CAA-3′. *M. bovis* DNA was visualised after a series of reactions in which a quencher was separated from a fluorophore after which a fluorescent signal was measured with the ABI-7500 machine. *M. bovis* DNA was considered to be present at a cycle threshold (Ct) value of $\leq$ 42. The lower the Ct-value, the higher the concentration of *M. bovis* DNA in the sample, categorised as – (Ct >42), and 1 + to 5 + (increasing amounts of *M. bovis* present. Ct < 42: 1, Ct < 40: 2, Ct < 38: 3, Ct< 35: 4 and Ct <32: 5). Samples with Ct-value > 40 (category 1 +) were considered outside the linearity range and below the lower limit of PCR quantification; therefore, these results had a higher measurement uncertainty and were interpreted with caution.

For *Mycoplasma* culture, individual milk (10 μL) or BTM (100 μL) were dispensed onto pleuropneumonia-like organism (PPLO) agar, which is a standard *Mycoplasma* medium used in many laboratories .[1] Inoculated PPLO-agar plates were incubated under normal atmospheric conditions but higher humidity (100%) for a maximum of 10 days at 37 °C. The growth of typical micro-colonies was assessed on days 3, 6 and 10 with use of a dissecting microscope.

2.4. Sample size

Sample size for detection of disease in the affected farms was calculated using the following assumptions: average Dutch farm (100 dairy cattle, 25 young stock and 15 calves); an outbreak in which 20% of animals showed seroconversion (assumption based on previous experience with outbreaks in the Netherlands), the calculated aimed sample sizes were 13 dairy cows, 13 young stock and 10 calves. This design ensured the ability to demonstrate the transmission of *M. bovis* with 95% confidence (WinEpi) .[2]

2.5. Statistical analysis

The differences in prevalence in the age groups were examined across time using logistic regression models. Data were corrected for the fact that animals within the same farm are more similar than animals at different farms. Descriptions of the outbreak situation per farm at the first visit (v0; Table 1) were provided. It was recorded where the animals were housed at the start of infection (as far as possible), the extent to which healthy animals in all age groups became infected, based on ELISA, PCR and, if applicable, culturing results, the extent in which test results from clinically suspected animals contained *M. bovis* antibodies during the course of the study and the pathways and risk factors that may have played a role. All analyses were performed using STATA/SE version 14 software. The error of the presence of *M. bovis* found in the previous period was modelled using a general estimation equation (repeated option in PROC GENMOD, SAS, version 9.4).

3. Results

3.1. Descriptive statistics

Descriptive statistics of the 20 farms are listed in Table 1. Twelve of these herds (60%) experienced both mastitis and arthritis in dairy cattle. Five herds experienced clinical arthritis and mastitis, while *M. bovis* was only found in bulk milk. One herd experienced no mastitis during the outbreak and three herds had only *M. bovis* associated mastitis without arthritis. Two herds experienced respiratory signs in dairy cows and four herds respiratory signs in calves.

The number of clinically suspected dairy cows per farm varied from 1% to more than 10%. The farms were spread over the Netherlands. The average farm size (*n* minimum, maximum) was 30 calves (10, 129), 67

[1] See: Laboratory Handbook on Bovine Mastitis, NMC, 2017 page 119. https://www.academia.edu/50651208/Laboratory_Handbook_on_Bovine_Mastitis (Accessed 30 April 2022)

[2] See: WinEpi: Working IN EPIdemiology. http://www.winepi.net/uk/sample/indice.htm. (Accessed 30 April 2022).

Table 1
Descriptive statistics of the 20 farms with an acute clinical M. bovis outbreak.

Farm ID	*M. bovis*-associated clinical signs in dairy cows	No. of animals per age group (V_0)			Purchase of cattle over the preceding 5 years	Mean milk production characteristics		
		1–6 months	7–24 months	> 24 months		kg milk/day	Fat %	Protein %
A	A, M	20	58	117	Yes	29.9	4.29	3.51
B	A, M	61	97	231	Yes	23.5	4.44	3.74
C	A, M	13	46	96	No	26.6	4.33	3.49
D	A, M	12	12	60	No	22.2	4.60	3.62
E	A, M	20	69	140	Yes	27.2	4.76	3.56
G	A, M	14	80	123	No	30.2	4.67	3.54
I	A, M	129	253	572	Yes	29.3	4.37	3.53
L	A, M	17	32	51	No	30.7	4.48	3.48
N	A, M	22	58	102	Yes	26.0	4.55	3.60
R	A, M	40	78	254	Yes	29.6	4.32	3.47
S	A, M	12	32	92	No	28.9	4.19	3.60
T	A, M	18	48	129	Yes	27.9	4.43	3.57
J	A, (M)	43	85	251	Yes	27.5	4.54	3.46
O	A, (M)[a, b and c]	26	74	166	Yes	28.9	4.28	3.45
M	A, (M)	22	46	165	Yes	30.0	4.20	3.53
P	A, (M)	63	118	325	Yes	28.6	4.52	3.72
H	M	28	66	147	No	25.6	4.01	3.46
F	M	18	46	143	Yes	25.9	4.49	3.48
K	M	10	17	50	No	30.1	3.87	3.42
Q	A	10	29	92	No	23.2	4.49	3.65

ID, Identification; V0, first visit; A, arthritis; M, mastitis.
[a] Milk samples of individual cows and bulk milk were all culture-negative (i.e. M. bovis associated mastitis was not demonstrated).
[b] Individual milk samples of all M. bovis suspected cows were all culture-negative, but individual milk samples from different randomly selected cows and BTM samples were culture-positive.
[c] Individual milk samples were all culture-negative, but BTM samples were culture-positive.

young stock (12, 253) and 165 dairy cows (50, 572) per farm. Over the last 5 years, 12 farms introduced animals into the herds. The average milk production was 27.6 kg milk/day with 4.39% fat and 3.54% protein content.

In total, 116 clinically suspected and 808 healthy animals were sampled at v0. These numbers decreased to 89 clinically suspected

Table 2
ELISA results (serum) and PCR results (conjunctival fluid) from clinically suspected animals [a] and healthy cattle by age group per round (grouped into 0 = 'negative', 1/2 + = low 'positive' and 3/4/5 + = high 'positive'), including total number of samples.

Age group	Sampling time	ELISA results			PCR results			Number of samples
		0	1/2 +	3/4/5 +	0	1/2 +	3/4/5 +	
Clinically suspected cattle[1]	V_0	41.9%	22.6%	35.5%	34.8%	35.9%	29.3%	93
	V_2	45.8%	27.7%	26.5%	38.1%	36.9%	25.0%	83
	V_5	54.0%	28.7%	17.2%	71.3%	23.0%	5.7%	87
	V_8	67.8%	24.1%	8.0%	71.3%	21.8%	6.9%	87
	V_{11}	72.3%	22.9%	4.8%	81.9%	14.5%	3.6%	83
	Average	56.1%	25.2%	18.7%	59.1%	26.6%	14.3%	433
Healthy dairy cows	V_0	52.3%	31.6%	16.2%	34.1%	41.3%	24.6%	266
	V_2	57.1%	32.3%	10.5%	38.2%	43.4%	18.4%	266
	V_5	65.8%	27.4%	6.8%	70.3%	24.0%	5.7%	263
	V_8	74.7%	21.5%	3.8%	69.8%	20.4%	9.8%	265
	V_{11}	80.1%	18.8%	1.1%	77.8%	18.4%	3.8%	261
	Average	65.9%	26.3%	7.7%	58.0%	29.5%	12.5%	1321
Healthy young stock	V_0	73.8%	20.7%	5.5%	66.0%	23.8%	10.2%	256
	V_2	67.8%	27.1%	5.1%	71.4%	21.2%	7.5%	255
	V_5	76.5%	16.9%	6.7%	81.6%	11.8%	6.7%	255
	V_8	80.0%	17.3%	2.7%	76.5%	17.6%	5.9%	255
	V_{11}	81.3%	16.8%	2.0%	80.1%	14.1%	5.9%	256
	Average	75.9%	19.7%	4.4%	75.1%	17.7%	7.2%	1277
Healthy calves	V_0	76.5%	18.7%	4.8%	43.3%	33.2%	23.5%	187
	V_2	70.7%	23.9%	5.4%	55.4%	29.9%	14.7%	184
	V_5	70.7%	25.5%	3.7%	73.4%	19.1%	7.4%	188
	V_8	60.8%	34.4%	4.8%	71.4%	22.2%	6.3%	189
	V_{11}	58.0%	36.7%	5.3%	75.0%	18.6%	6.4%	188
	Average	67.3%	27.9%	4.8%	63.8%	24.6%	11.6%	936
Healthy cattle (total)	V_0	66.4%	24.3%	9.3%	48.1%	32.8%	19.1%	709
	V_2	64.5%	28.2%	7.2%	54.7%	31.9%	13.5%	705
	V_5	71.0%	23.1%	5.9%	75.2%	18.3%	6.5%	706
	V_8	72.9%	23.4%	3.7%	72.6%	19.9%	7.5%	709
	V_{11}	74.6%	22.8%	2.6%	77.9%	16.9%	5.2%	705
	Average	69.9%	24.4%	5.7%	65.7%	23.9%	10.4%	3534

V, visit number, e.g., V0, first visit etc.
[a]90% of clinically suspected animals were dairy cows (388/433 samples).

animals and 784 healthy at v11, mainly due to removal of cows. In total 9625 samples were collected and animals were sampled on average 4.3 times for blood and conjunctival fluid and 3.7 times for milk.

3.1.1. *Mycoplasma bovis transmission within age groups over time*

From the time an outbreak was confirmed, *M. bovis* infection was not only present in dairy cows, but also in young stock and calves (80% of the farms).

3.1.2. *Dairy cows*

At v0, 58.1% of clinically suspected and 47.8% of healthy dairy cows were ELISA-positive (1 +−5 +; Table 2). The serological response decreased over time in both clinically suspected and healthy cows (see Fig. 1A), but did not reach zero. Seroprevalence dropped over time (v5 to v11) from 45.9% to 27.7% for clinically suspected and from 34.2% to 19.9% for healthy dairy cows (Table 2). This decrease was highest in the groups with the strongest reaction (> 2 +). Within dairy cows ELISA-levels were correlated in three consecutive visits while in the remaining age groups ELISA-levels were highly correlated in two consecutive visits.

Overall herds a slight decrease in PCR 'positive' tests was seen in the first three visits, followed by a slight increase between v5 and v8 (Fig. 1A). At v0, 65.2% and 65.9% of clinically suspected and healthy dairy cows, respectively tested 'positive' for the presence of *M. bovis* DNA in the conjunctival fluid (Table 2). Among healthy cows these figures decreased to 29.7% at v5 and 22.2% at v11 (Table 2). The percentage of 'positive' results at v0 was higher in cows than in youngstock and calves, but the prevalence was comparable for all age groups at v11. A large proportion of samples at v11 contained low levels (1 +, 2 +) of *M. bovis* DNA most probably a reflection of low levels of *M. bovis* DNA in the farm environment. Equal amounts of low positivity were observed when comparing clinically suspected and healthy cows ($P = 0.40$). Stronger PCR 'positivity' (3 +, 4 + or 5 +) was more often demonstrated in clinically suspected animals (14.3%) compared to healthy animals (10.4%; $P = 0.03$). As with serology, the percentages of strong responders (> 2 +) showed the steepest decline over time, a finding also observed in the other groups. The correlation between the PCR-levels at consecutive sampling periods within age groups was minimal.

3.1.3. *Young stock*

Young stock showed at v0 a seroprevalence of 26.2%, which increased to 32.2% at v2 and decreased to 18.7% at v11. The prevalence of positive conjunctival fluid PCR in young stock was 34.0% at v0 and decreased to 20.0% at v11 (Table 2 and Fig. 1B).

3.1.4. *Calves*

On average, 32.7% of the calves showed a serological response which increased from 23.5% (v0) to 42.0% (v11). An noticeable increase to 39.2% occurred at v8 mainly due to the increasing number of low positive (< 2 +) samples (Table 2). The percentage of PCR positive conjunctival fluid samples showed a different pattern over time. At v0, 56.7% of the healthy calves were PCR positive which decreased to 25.0% at v11 (Fig. 1C).

3.2. *Mycoplasma bovis transmission between age groups over time*

The serological responses of dairy cows, young stock and calves were not correlated within farms at the various sample times, but the

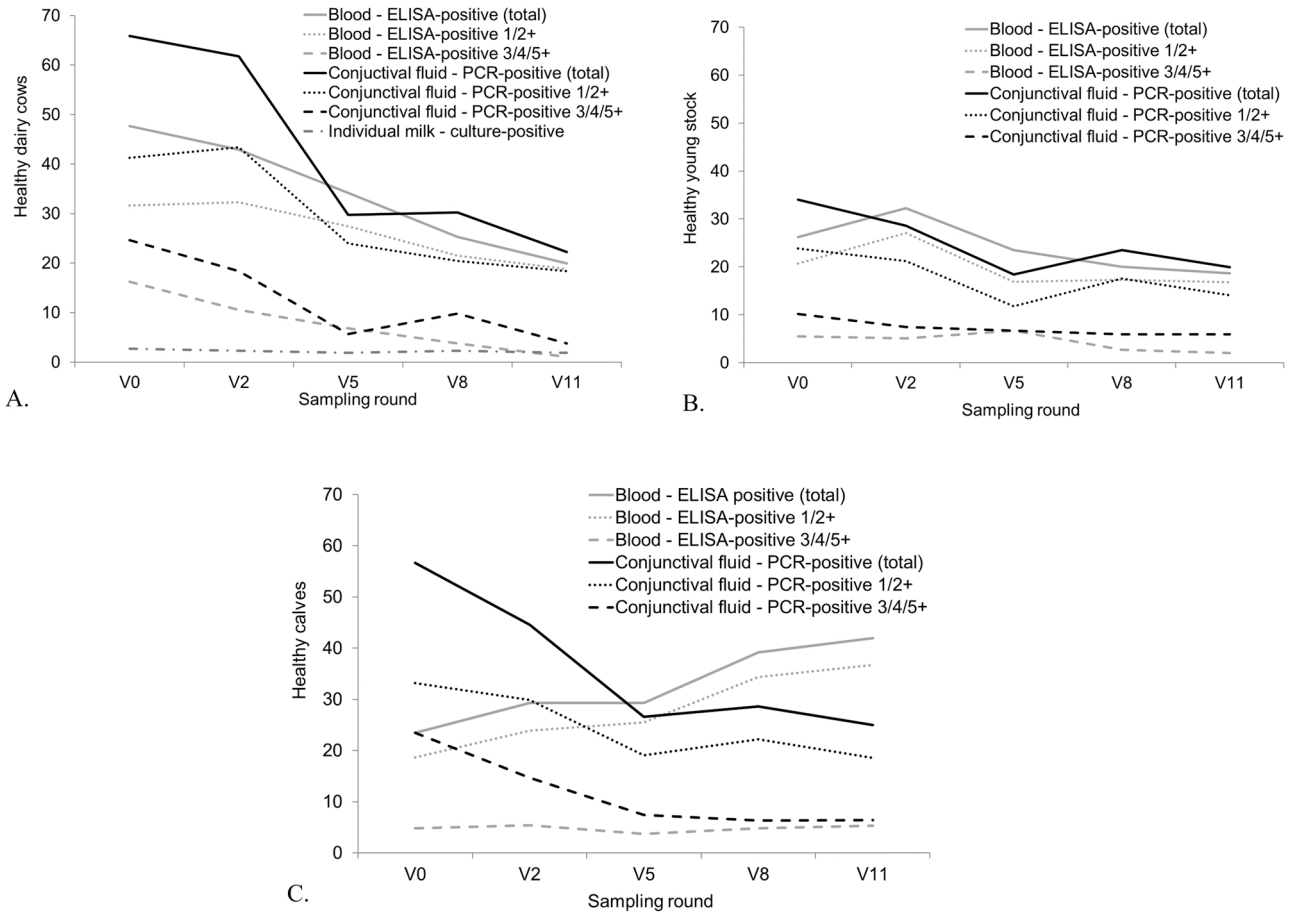

Fig. 1. A - C. *M. bovis* prevalence in healthy dairy cows (A), young stock (B) and calves (C) on outbreak farms based on ELISA (serum), PCR (conjunctival fluid) and, if applicable, culture (individual milk samples). V, visit number e.g., V0, first visit, V2, visit at week 2, etc.

Table 3
Mycoplasma bovis PCR test results of environmental samples at v0 and v11, grouped by farm and age-group. The numbers 0–5 indicate the amounts of M. bovis DNA detected (0 = 'negative', 1/2 = low 'positive' and 3/4/5 = high 'positive').

Farm	-Drinking buckets calves	-Water troughs calves 1–6 mo.	-Water troughs young stock 7–12 mo.	-Water troughs young stock 12–24 mo.	-Water troughs dairy cows	-Lying cubicles dairy cows	-Water troughs dry cows	-Drinking buckets calves	-Water troughs calves 1–6 mo.	-Water troughs young stock 7–12 mo.	-Water troughs young stock 12–24 mo.	-Water troughs dairy cows	-Lying cubicles dairy cows	-Water troughs dry cows
	Sampling time v0							Sampling time v11						
A	2	3	0	0	2	2	1	3	1	0	0	1	0	0
B	5	4	3	3	4	3	3	0	0	0	0	0	1	0
C	2	2	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0		0	2	0	0	0	0	0	0	0	0
E	0	0	0	0	2	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	1	0	0	0	0	0	0	0
H	1	0	0	0	0	0	0	0	0	2	2	2	0	3
I	1	0	0	0	1	3	3	0	1	1	0	0	0	0
J	0	2	1	0	0	0	0	0	0	3	0	0	0	0
K	1	0	3	0	1	1	0	1	0	0	0	0	0	0
L	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M	3	0	0	0	1	0	0	0	0	0	0	0	0	0
N	2	1	0	1	1	1	0	3	0	0	0	0	0	0
O	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P	2	3	1	0	1	0	3	0	0	0	0	0	0	0
Q	0	0	0	0	0	0	0	1	1	0	0	0	0	
R	4	3	0	0	1	0	0	0	0	0	0	0	0	0
S	0	0	0	0	3	0	0	0	1	0	0	0	0	0
T	0	0	0	0	0	3	4	0	0	0	0	0	0	0

V0, first visit; V11, visit at week 11.

correlations of ELISA results between calves and young cattle showed a trend ($r2 = 0.39$; $P = 0.09$). Correlation between the results of dairy cows and those of the other groups were not observed.

Based on PCR-results, the numbers of infected calves were correlated to that of young stock when comparing across visits. The *M. bovis* PCR ct counts in calves and young stock were highly correlated in all visits ($r_{total} = 0.81$; $P < 0.01$). No correlations between the *M. bovis* PCR-levels of dairy cows and those of calves, dairy cows and young stock were seen.

3.3. Environmental samples

M. bovis DNA was detected in at least one environmental sample in 80% of the farms at v0 and in 45% of farms at v11 (Table 3).

3.4. Milk samples

Most farms (17/20) had at least one milk sample in which *Mycoplasma* was cultured (i.e., 15 farms with positive individual milk samples and 12 farms with positive BTM samples). *Mycoplasma* was cultured in 54/1692 individual milk samples. Over the entire period, *Mycoplasma* was cultivated in milk in an average of 6.5% of clinically suspected and 2.2% of healthy dairy cows (Table 4). The prevalence of culture-positive milk samples from clinically suspected dairy cows ($n = 50$) dropped from 14.5% (v0) to 3.8% at v5 and 5.3% at v11. The prevalence of culture-positive milk samples from healthy dairy cows remained the same during the investigation (1.9–2.7%). Healthy dairy cows with culture-'positive' milk samples at v0 were more often culture-positive at v11 ($r = 0.70$; $P < 0.01$). At v0, the BTM was *Mycoplasma* culture-positive in 50% of the farms; the percentage decreased to 15% at v11.

At the end of the three-month study period (v11), at least one serum, conjunctival fluid or milk sample tested positive in 100%, 90% and 30% of the farms, respectively.

4. Discussion

Selected farms were associated with an early-phase *M. bovis* outbreak, that was expected to enable the monitoring of transmission within and between animal age groups. The detection of a *M. bovis* outbreak was based on the observations of the farmer, clinical investigation by the practicing veterinarian and a laboratorial diagnosis. However, based on test results, it was apparent that only four farms (A, I, Q and T) had no dissemination of *M. bovis* to other age groups at v0 yet and had possibly experienced an acute outbreak at the initiation of the sampling period. In all remaining farms the prevalence of ELISA-positive cows was already highest at v0, which may imply that *Mycoplasma* was already present in the farms. These results were not correlated with the farmer's or veterinarian's awareness of clinical signs, enabling the

Table 4
Mycoplasma culture results in percentages of individual milk samples of the clinically suspected dairy cows and healthy dairy cows during the five visits to investigate the persistence and transmission of the bacteria after introduction.

Animal group	Sampling time	Culture result		Number of samples
		Negative	Positive	
Clinically suspected dairy cows	v_0	85.5%	14.5%	83
	v_2	96.0%	4.0%	75
	v_5	96.0%	4.0%	75
	v_8	96.2%	3.8%	78
	v_{11}	94.7%	5.3%	75
	Average	93.5%	6.5%	
Healthy dairy cows	v0	97.3%	2.7%	257
	v2	97.7%	2.3%	265
	v5	98.1%	1.9%	262
	v8	97.7%	2.3%	263
	v11	98.1%	1.9%	259
	Average	97.7%	2.2%	

V0, first visit; V2, visit at week 2, etc.

infections to spread substantially among dairy cows and further. Introduction may have also occurred in the other age groups which may have been obscured by the inclusion criteria used in the study.

A serological response was found before the last visit at v11, in line with our expectations and those of others, that seroconversion takes 2–3 weeks following exposure (Wawegama et al., 2014). Andersson et al. 2019 showed that the indirect Elisa kit used for this study had a lower sensitivity than others in a recent trial, suggesting the results in this study may have underestimated the true number of sero-positive samples. Due to intermittent excretion and loss of bacteria during sample transfer to the laboratory more cows were serologically positive than culture-positive (Nunez et al., 2008; Akan et al., 2014). The absence of *Mycoplasma*-related clinical signs in infected cattle is in line with a case report about a large dairy herd. Specifically, 13 cows had confirmed infection, among which only eight cows exhibited arthritis, and one had mastitis (Punyapornwithaya et al., 2011). Seroconversion of asymptomatic cows indicated that some cattle experienced *M. bovis* infection or became exposed without developing disease, an occurring that is not abnormal for infectious diseases (Morris et al., 1994).

Based on the PCR results, *M. bovis* was found at a comparable level in conjunctival samples of both clinically suspected and healthy animals. Via the nasolacrimal duct, lacrimal fluid is in direct contact with the mucous membranes in the upper part of the respiratory tract, where *Mycoplasma* resides. This may result in the presence of *M. bovis* in the conjunctival fluid and may be the reason for which this location is suggested as predilection site for *Mycoplasma* diagnostic samples (Maeda et al., 2003; Kleinschmidt et al., 2013; Oliveira et al., 2019). The *M. bovis* PCR levels of young stock and calves were highly correlated in all visits, indicating that calves and young stock were likely to be infected at the same level at the same time.

Contrary to general recommendations *Mycoplasma* was cultured under ambient CO_2 concentrations which could have given some false-negative results. However, *M. bovis* is known to be relatively insensitive to CO_2 concentration differences (Gourlay et al., 1979; Lowe et al., 2018). Lowe et al. (2018) discussed the evidence for CO_2 enrichment and suggested that the use of ambient CO_2 conditions might be an advantage for culture. Finally, in routine cultures of suspected calf pneumonia cases performed for more than 10 years, more than 50% positive results were observed, confirming that successful culture is feasible under ambient CO_2 conditions. For these reasons, unsuccessful culture was not viewed as a significant cause of false negative results. *Mycoplasma* spp. positive cultures were not confirmed by sequence analysis and could also imply other mycoplasma spp., such as *Mycoplasma bovirhinis, Mycoplasma bovigenitalium, Mycoplasma bovoculi* or *Acholeplasma spp.* (Parker et al., 2018). However, the literature supports that *M. bovis* is the most important and frequently isolated *Mycoplasma* spp. associated with disease in cattle worldwide (Panciera and Confer, 2010; Fox, 2012).

Remarkably, only two herds experienced respiratory signs in a few dairy cows and four herds in calves, given aerogenic transmission is thought to be the main route of transmission in both calves and mature dairy cattle (Nicholas et al., 2016).

This study showed transmission between age groups in varying degrees in all farms. *M. bovis* could spread easily between young stock and calves. *M. bovis* infection in calves was correlated with that of young stock, which may indicate easy transmission between these groups. This could be due to direct contact, transmission by employees, and introduction of young animals into the older age groups. This is consistent with previous Swiss study in which transmission was correlated with contaminated milk. The mechanism of transmission was not investigated in our study. Whether the decreasing rate of infection was a consequence of a removal of active shedders or other farm measures, remains unclear (Aebi et al., 2015). An earlier study from Washington State University observed that the immediate culling of *Mycoplasma*-related mastitis cows was not associated with an accelerated end to new infections (Punyapornwithaya,et al., 2010, 2012). A factor that influenced transmission was feeding pooled colostrum or raw mastitis milk to calves (Gille et al., 2020), which was practised in approximately 30% of herds investigated in this study.

To the best of our knowledge, this is the first study that aimed to closely monitor acute clinical *M. bovis* outbreaks in such a large number of farms (20). The results supported the hypotheses that *M. bovis* infects all age groups during the early outbreak stage despite separate housing and that animals, not exhibiting clinical signs, may nevertheless carry *M. bovis* bacteria. Findings also demonstrate that *M. bovis* is not likely to be eradicated from an outbreak farm within 3 months after the onset of clinical signs. Consistent with a previously published report (Nicholas et al., 2016) this study indicates that a *Mycoplasma* infection is clinically self-limiting at the herd level in most cases. However, disease patterns after the 3-month investigation periods are unknown.

5. Conclusions

Based on clinical and laboratory investigations the main conclusions of this study of the dynamics of *M. bovis* in Dutch dairy herds experiencing an acute clinical outbreak was that the infection was already dispersed throughout the farms. The occurrence and course of clinical signs were ostensibly unpredictable; despite the apparent presence of the agent 3 months after the start of the clinical outbreak, hardly any new clinical problems were observed.

Conflict of interest statement

This study was financially supported by the Dutch Dairy Industry (Zuivel NL), but the organisation did not play any role in the study design or in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication. The authors do not have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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