



Within-herd transmission of *Mycoplasma bovis* infections after initial detection in dairy cows

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ABSTRACT

Mycoplasma bovis outbreaks in cattle, including pathogen spread between age groups, are not well understood. Our objective was to estimate within-herd transmission across adult dairy cows, youngstock, and calves. Results from 3 tests (PCR, ELISA, and culture) per cow and 2 tests (PCR and ELISA) per youngstock and calf were used in an age-stratified susceptible-infected-removed/recovered (SIR) model to estimate within-herd transmission parameters, pathways, and potential effects of farm management practices. A cohort of adult cows, youngstock, and calves on 20 Dutch dairy farms with a clinical outbreak of *M. bovis* in adult cows were sampled, with collection of blood, conjunctival fluid, and milk from cows, and blood and conjunctival fluid from calves and youngstock, 5 times over a time span of 12 wk. Any individual with at least one positive laboratory test was considered *M. bovis*-positive. Transmission dynamics were modeled using an age-stratified SIR model featuring 3 age strata. Associations with farm management practices were explored using Fisher's exact tests and Poisson regression. Estimated transmission parameters were highly variable among herds and cattle age groups. Notably, transmission from cows to cows, youngstock, or to calves was associated with R-values ranging from 1.0 to 80 secondarily infected cows per herd, 1.2 to 38 secondarily infected youngstock per herd, and 0.1 to 91 secondarily infected calves per herd, respectively. In case of transmission from youngstock to youngstock, calves or to cows, R-values were 0.7 to 96 secondarily infected youngstock per herd, 1.1 to 76 secondarily infected calves per herd, and 0.1 to 107 secondarily infected cows per herd. For transmission from calves to calves, youngstock or to cows, R-values were

0.5 to 60 secondarily infected calves per herd, 1.1 to 41 secondarily infected youngstock per herd, and 0.1 to 47 secondarily infected cows per herd. Among on-farm transmission pathways, cow-to-youngstock, cow-to-calf, and cow-to-cow were identified as most significant contributors, with calf-to-calf and calf-to-youngstock also having noteworthy roles. Youngstock-to-youngstock was also implicated, albeit to a lesser extent. Whereas the primary focus was a clinical outbreak of *M. bovis* among adult dairy cows, it was evident that transmission extended to calves and youngstock, contributing to overall spread. Factors influencing transmission and specific transmission pathways were associated with internal biosecurity (separate caretakers for various age groups, number of people involved), external biosecurity (contractors, external employees), as well as indirect transmission routes (number of feed and water stations).

Key words: *Mycoplasma bovis*, dairy farms, transmission, susceptible-infected-removed/recovered models, infection dynamics

INTRODUCTION

Mycoplasma bovis causes huge economic losses in the cattle industry (Nicholas and Ayling, 2003). *Mycoplasma bovis* is a major contagious mastitis pathogen, but it can also cause pneumonia, arthritis, keratoconjunctivitis, otitis media, abortion, infertility, and subcutaneous abscesses (González and Wilson, 2003). *Mycoplasma bovis* can colonize mammary glands, joints, tendon sheaths, and periarticular tissues, causing clinical or subclinical disease (Maunsell et al., 2011). *Mycoplasma* spp. lack a cell wall (Maunsell et al., 2011; González and Wilson, 2003), conferring natural resistance to antimicrobials targeting cell wall synthesis (González and Wilson, 2003).

Mycoplasma bovis can affect calves, youngstock, and adult dairy cows (Vähänikkilä et al., 2019). Although

Received February 21, 2023.

Accepted August 21, 2023.

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factors affecting development of clinical signs in infected cattle are not fully elucidated, stress-related immunosuppression has been speculated to increase the likelihood of clinical disease (Aebi et al., 2015). Regarding transmission dynamics, direct spread between cows is known to occur during milking or via nose-to-nose contact (González and Wilson, 2003; Punyapornwithaya et al., 2011). However, indirect transmission is also possible via communal drinking and feeding troughs (Pennerman et al., 2022). In addition, *M. bovis* can survive for months in sand under cool and moist conditions (Justice-Allen et al., 2010).

Diagnosis of *M. bovis* is complicated by inconsistent disease expression and intermittent shedding (Byrne et al., 2000; Biddle et al., 2003; Maunsell et al., 2011; Hazelton et al., 2018). Classical diagnostics involve bacteriological culture of body fluids such as milk, joint fluids, or eye or nasal swabs (Petersen et al., 2018a). However, culture is complex and time-consuming, exhibits low sensitivity and, in certain instances, reduces specificity. Moreover, frozen storage considerably decreases sensitivity (Biddle et al., 2004; Pinnow et al., 2001). Recently developed PCR tests had comparable sensitivity and specificity to culture, with PCR performing relatively better than culture on frozen milk and clinical samples (Pinnow et al., 2001). Antibody ELISA are also available but mainly detect antibodies post-exposure, rather than indicating current or active infections (Szacawa et al., 2015). During outbreaks, individual antibody responses are dynamic and vary among cows (Byrne et al., 2005; Petersen et al., 2018a), and antibody titers generally decline within 8 to 9 wk after the onset of clinical signs (Byrne et al., 2000; Petersen et al., 2018a). Nonetheless, infected cows may shed *M. bovis* for several months (Punyapornwithaya et al., 2011; Hazelton et al., 2018). Herd-level diagnostics require sequential testing of multiple animals using serology and PCR (Szacawa et al., 2015; Vähänikkilä et al., 2019). However, optimal combinations of diagnostic tests remain uncertain, and detailed knowledge regarding how *M. bovis* spreads within and between various age groups is currently lacking.

On 20 Dutch dairy farms with a clinical outbreak of *M. bovis* in adult cows, our objectives were to: (1) estimate within-herd transmission between adult dairy cows, youngstock, and calves using an age-stratified susceptible-infected-removed/recovered (SIR) model and 2 or 3 individual test results (PCR, ELISA, and culture); (2) identify the most likely within-herd transmission pathways between age groups; and (3) explore potential explanatory farm management factors associated with transmission parameters and pathways.

MATERIALS AND METHODS

Herds

Longitudinal data were collected in 20 Dutch dairy herds, all of which experienced outbreaks of clinical *M. bovis* mastitis or arthritis in adult cows between February 2016 and April 2017. Each herd was intensively sampled 5 times over 3 mo after the first clinical signs appeared. This study was approved by the Dutch Central Commission of Animal Studies (CCD, number: 2015300) and the Institution for Animal Welfare of Royal GD Animal Health (Deventer, the Netherlands).

Herds with cows having clinical signs of *M. bovis* mastitis (characterized by poor or spontaneous cessation of milk production or poor response to mastitis treatment), or any signs of arthritis, were recruited by informing Dutch veterinarians of the study through the veterinary newsletter of Royal GD, the online knowledge platform of Royal GD, and by *Mycoplasma* workshops for veterinary practitioners across the country. Also, farmers or veterinarians managing herds with clinical signs consistent with *M. bovis* that contacted the Royal GD consultancy phone desk Veekijker, an enhanced passive surveillance reporting system, were invited to participate (Vredenberg et al., 2022).

Herds were deemed eligible for inclusion if the farmer or veterinarian observed clinical signs of *M. bovis* within a maximum of 2 wk before notifying the outbreak. Within this 2-wk timeframe, at least 5 cows needed to be affected by clinical mastitis or arthritis. Herds with a known history of *M. bovis* in the past year were excluded. Eligible herds were required to submit either samples of milk (clinical mastitis) or blood (arthritis) obtained from 5 adult cows with clinical signs of *M. bovis* infection. These samples were subjected to testing to detect the presence of *M. bovis* through PCR, culture, or antibody ELISA methods. At least one test needed to yield a *M. bovis*-positive result, and this positive result should have occurred within the past 2 wk, as herds with a known history were excluded. Additional inclusion criteria were that herds: (1) needed to be a DHIA participant; (2) had >50 adult cows including dry cows; and (3) had sufficient head gates to restrain cows for sampling. Twenty herds met these inclusion criteria and were included in the study.

Sampling

Each herd was visited within 1 wk after confirmation of *M. bovis*. At this visit (T_0), the study protocol was explained in detail to the farmer, a risk assessment was performed, and samples collected (Figure 1). Sub-

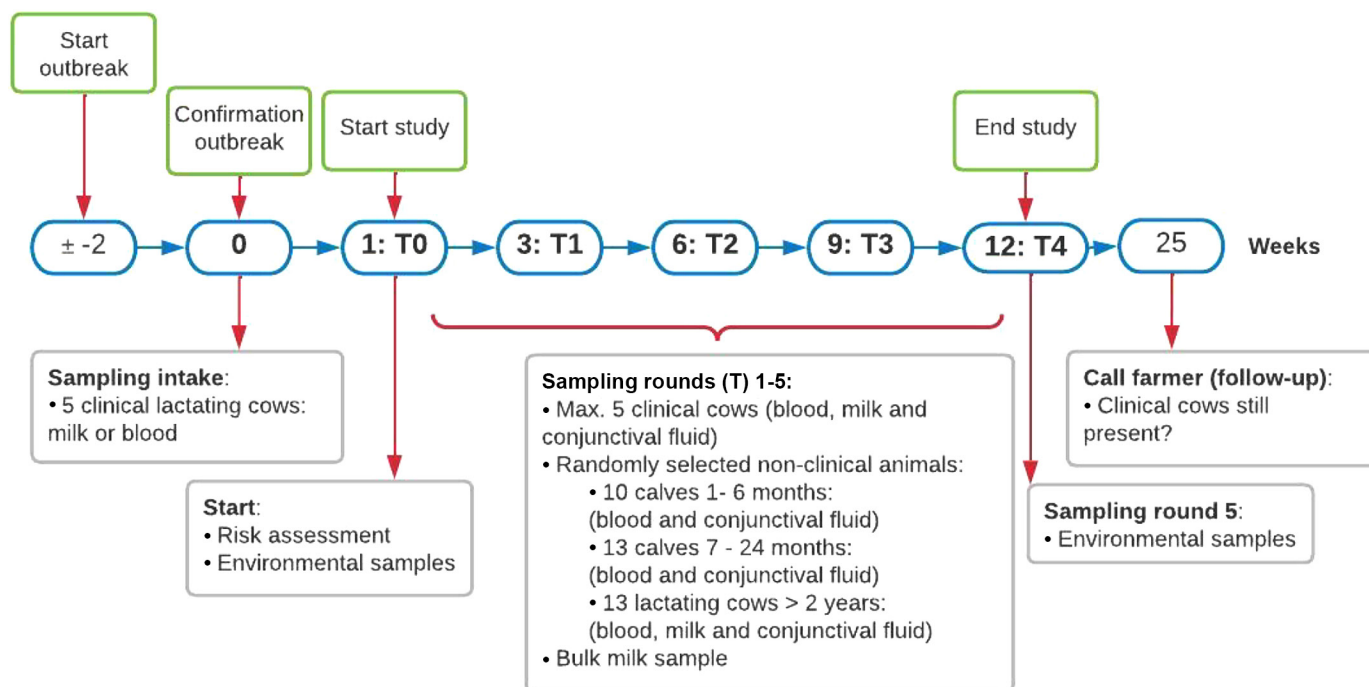


Figure 1. Sample and data collection at the 20 participating Dutch dairy farms to be used in an age-stratified susceptible-infected-removed/recovered (SIR) model to estimate within-herd transmission parameters. Collected samples included blood for antibody ELISA, milk (4-quarter sample) and bulk milk for culture, tears (conjunctival fluid sample of both eyes), and environmental samples for PCR.

sequently, the herd was resampled 2, 5, 8, and 11 wk after the start of the study in wk 1 (T_1 , T_2 , T_3 , and T_4 , respectively).

At each of the 5 sampling points, milk, blood, and conjunctival fluid samples were collected from a maximum of 5 adult cows that had clinical signs of *M. bovis* infection. If 5 cows were identified at T_0 with clinical signs, they were sampled throughout the study. If at T_0 <5 cows had clinical signs, newly appearing clinical cows could be included at any time point up to a maximum of 5 samples cases per time point. In addition, at any time point if any previously sampled cow was no longer present in the herd, it could be replaced by a new clinical case. At the same time, milk, blood, and conjunctival fluid samples were collected from a cohort of randomly preselected nonclinical adult cows, and blood and conjunctival fluid samples of youngstock and calves before the first visit at each sampling using runiform (Stata14/SE; StataCorp) with cattle having the lowest assigned number being included. The size of this cohort was based on proving the presence of *M. bovis* in a herd with 95% confidence, assuming an expected seroconversion of 20% of cattle within a herd and an average herd size of 100 cows, 15 youngstock, and 15 calves. The cohort consisted of 10 calves (1–6 mo), 13 youngstock (7–24 mo), and 13 adult cows (>24

mo). The same cohort was sampled throughout the study. In case of removal from the herd (culling due to disease, natural death, or being sold), the next nonclinical animal with the lowest randomly assigned number was selected and included in the cohort.

A conjunctival fluid sample was collected from the medial canthus of both eyes with a dry sterile flocculated swab (Eswab, Copan, Italy). Blood samples were collected from coccygeal vessels in a 10-mL vacutainer tube, and for lactating cows only, milk samples were aseptically collected from all 4 udder quarters into a sterile 10-mL tube. All samples were cold-stored in a cooler and immediately sent per herd batch to the laboratory of Royal GD Animal Health (GD) for analysis.

On each farm at T_1 , a risk assessment questionnaire was conducted, addressing the following topics: calf housing, youngstock housing, milking parlor, cow housing, pasture access, dry cow housing, calving pen, hospital pen, hygiene measures, immunosuppressive management factors, and miscellaneous (culling, recent management interventions, and common practices for handling *M. bovis*-diseased cows). The questionnaire (Supplemental Appendix S1, <https://doi.org/10.6084/m9.figshare.24520987.v1>; Biesheuvel, 2023a) was conducted by 2 experienced technicians (GD employees) specialized in udder health.

Laboratory Diagnostics

Culture (Individual Milk). Culture of individual milk samples for *Mycoplasma* was done in accordance with routine GD procedures, as described in Penterman et al. (2022). In short, 10 μ L of milk was fractionally spread on pleuropneumonia-like organism agar, a *Mycoplasma*-specific solid medium. The inoculated plate was incubated at 37°C under ambient CO₂ conditions. Then, 3, 6, and 10 d after incubation, the culture plate was microscopically examined for colonies typical of *M. bovis* (Middleton et al., 2017). Recently, it has become more evident that culturing *Mycoplasma* under very low conditions of CO₂ (candle jars) does not affect growth (Lowe et al., 2018).

ELISA (Blood). Detection of *Mycoplasma* antibodies in blood used a commercial indirect ELISA kit for *M. bovis* antibody detection (Bio-X K260 Diagnostics, Rochefort, Belgium). A detailed description is available in Penterman et al. (2022). In accordance with the manufacturer's recommendations, results were distinguished into 6 classes: negative, no antibodies (sample-to-positive percentage [S/P%] $\leq 37\%$), and positive, antibodies + (37% < S/P% $\leq 60\%$), ++ (60% < S/P% $\leq 83\%$), +++ (83% < S/P% $\leq 106\%$), ++++ (106% < S/P% $\leq 129\%$), and +++++ (S/P% > 129%), indicating increasing amounts of antibodies. Sensitivity and specificity of the ELISA (based on all positive classes) were estimated to be 14.1% (95% Bayesian credibility interval [BCI]: 11.6%–16.7%) and 97.2% (95% BCI: 95.9%–98.4%), respectively (Veldhuis et al., 2023).

PCR (Conjunctival Fluid Samples). Detection of *M. bovis* in conjunctival fluid samples used a KaspRT PCR, as described in Penterman et al. (2022). The cycle threshold (Ct) value was distinguished into 6 classes with increasing levels of *M. bovis* DNA in the sample: negative, no DNA (Ct > 42), and positive, DNA detected as + (Ct < 42), ++ (Ct < 40), +++ (Ct < 38), ++++ (Ct < 35), and +++++ (Ct < 32).

Infection Status. For each animal in the study, individual disease status was determined by aggregating the 2 or 3 laboratory results (culture, ELISA, and PCR) into 1 score. An animal was considered infected with *M. bovis* at a sampling day when at least one of the laboratory results had a positive outcome for *Mycoplasma*.

Data Analyses. Transmission dynamics of *M. bovis* were modeled in each of the 20 herds separately, based on the sampled cohort, using an age-stratified SIR model with 3 age strata: adult cows, youngstock, and calves. We made several assumptions: (1) a closed population (i.e., births and deaths were not considered over the 12-wk sampling period); (2) in the absence of robust evidence regarding latency, the latent period

was ignored in the model, meaning that it was assumed that infected cattle were infectious as soon as they were infected; and (3) recovered cattle are immediately immune and not susceptible to reinfection. For all cattle, this means that the recovery rate equaled the removal rate.

The SIR model designates each of the 3 cattle groups into 3 compartments: susceptible (S), infected (I), or removed/recovered (R). Letting $i \in \{L, Y, C\}$ denote adult cows, youngstock, and calves, respectively, S_i , I_i , and R_i provide the proportion of cattle in the indicated compartment in group i (Figure 2). No distinction between clinical and nonclinical cows was made in the adult cow group. In this model, all possible transmission pathways were allowed to be present. Infected cattle in each age group could infect cattle of the same or different age groups. Changes in the proportion of cattle between the 3 compartments over time for each age group (dS/dt , dI/dt , and dR/dt) were described by the following ordinary differential equations (Equation 1), where β_{ij} is the transmission rate from animals in group j to animals in group i , ($i, j \in \{L, Y, C\}$):

$$\frac{dS_L}{dt} = -(\beta_{LL}I_L + \beta_{LY}I_Y + \beta_{LC}I_C)S_L,$$

$$\frac{dS_Y}{dt} = -(\beta_{YY}I_Y + \beta_{YL}I_L + \beta_{YC}I_C)S_Y,$$

$$\frac{dS_C}{dt} = -(\beta_{CC}I_C + \beta_{CY}I_Y + \beta_{CL}I_L)S_C,$$

$$\frac{dI_L}{dt} = (\beta_{LL}I_L + \beta_{LY}I_Y + \beta_{LC}I_C)S_L - I_L,$$

$$\frac{dI_Y}{dt} = (\beta_{YY}I_Y + \beta_{YL}I_L + \beta_{YC}I_C)S_Y - I_Y,$$

$$\frac{dI_C}{dt} = (\beta_{CC}I_C + \beta_{CY}I_Y + \beta_{CL}I_L)S_C - I_C,$$

$$\frac{dR_L}{dt} = I_L,$$

$$\frac{dR_Y}{dt} = I_Y, \text{ and}$$

$$\frac{dR_C}{dt} = I_C. \quad [1]$$

This SIR model contained 10 parameters, the transmission rates, β_{ij} , and removal rate, γ . The transmis-

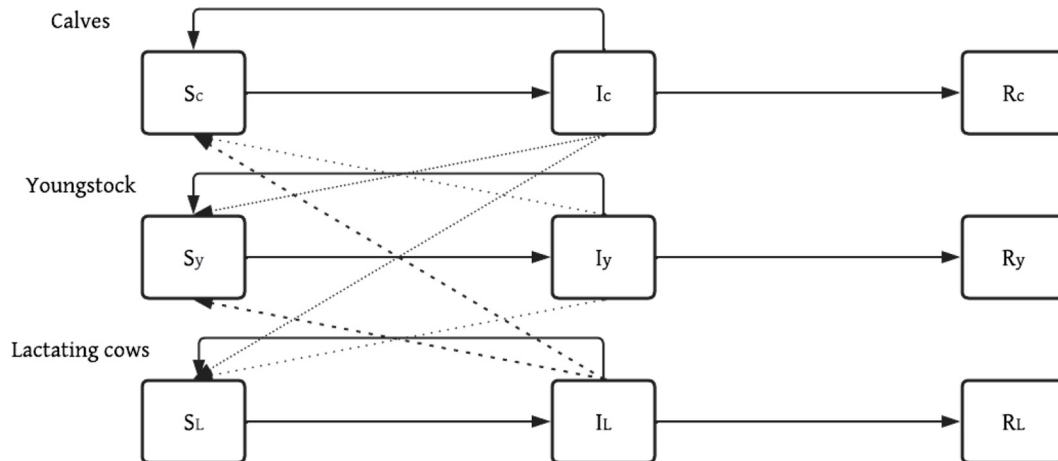


Figure 2. The SIR model (where S = susceptible, I = infected, and R = removed/recovered) for modeling *Mycoplasma bovis* transmission between calves (c), youngstock (y), and lactating adult cows (l).

sion rate defined the number of secondary infections in age group i caused by 1 infectious animal in age group j per unit of time in an otherwise fully susceptible herd and $1/\gamma$ provided the mean number of time units an animal is expected to remain infectious. These parameters were combined to determine the basic reproduction ratios (R-value = β_{ij}/γ) that yielded the average number of secondary infections in age group i caused by 1 infectious animal in age group j over the entire course of its infectious period in an otherwise fully susceptible herd (Diekmann et al., 1990). To determine the values for each β_{ij} and γ that best matched the fitted model to the observed data, a procedure in which the sum of squared errors of the observed and predicted number of infections over time is minimized was used. The function was optimized using various starting values to find the 9 β and 1 γ parameters that yielded an epidemic trajectory closest to the observed data. Data from the first sampling round (T_0) were used to determine initial values of S_i , I_i , and R_i .

Mycoplasma bovis was assumed not to have been introduced by purchased cows, youngstock, calves, or other external sources after notification of the initial outbreak; consequently, all transmission was deemed to be a result of the initial outbreak, and the infectious period was assumed to be the same across all 3 groups. Cattle were assumed not to move from 1 age group to another during the study. Within-cattle group transmission rates were estimated separately for adult cows, youngstock, and calves. In addition, between-cattle group transmission parameters were estimated (Figures 3 and 4).

Subsequently, it was determined on which farms each of the 9 transmission pathways occurred ($\beta > 0$). Furthermore, management factors obtained in the risk as-

essment were used in a herd-level univariable analysis to identify potential explanatory farm factors for the occurrence of each of the transmission pathways by performing a Fisher's Exact test for categorical variables or a Poisson regression for count variables, accounting for total number of adult cows on the farm (exposure). General trends in the dynamics of *M. bovis* transmission between and within age groups in dairy herds based on the same data are described in Penterman et al. (2022).

All data analyses were performed using the deSolve package in R Version 4.1.1 (Soetaert et al., 2010; R Core Team, 2021) and STATA/SE 16.1 (StataCorp, College Station, Texas). Results were considered significant at $P \leq 0.05$.

RESULTS

Herds and Cattle

In total, 972 cattle from 20 dairy herds were sampled. Herd size ranged from 50 to 572 cows (median = 140 cows), 12 to 253 youngstock (median = 58) and 10 to 129 calves (median = 20). A mean of 46 cattle (median = 45) per herd were sampled (range 31 to 68). Results obtained during the inclusion process were excluded from the analysis resulting in 924 remaining cattle. Adult cows (cows >24 mo) were the largest group of sampled cattle at any of the 5 samplings ($n = 454$, 49.1%), followed by youngstock between 6 and 24 mo ($n = 269$, 29.1%) and calves <6 mo ($n = 201$, 21.8%).

Median number of samples per animal was 5 (range, 1 to 5 samples). Eighty-four cattle that were initially selected to be sampled dropped out of the study (adult cows $n = 75$, youngstock $n = 4$, calves $n = 5$). Culling or death occurred for 65% ($n = 49$) of removed adult

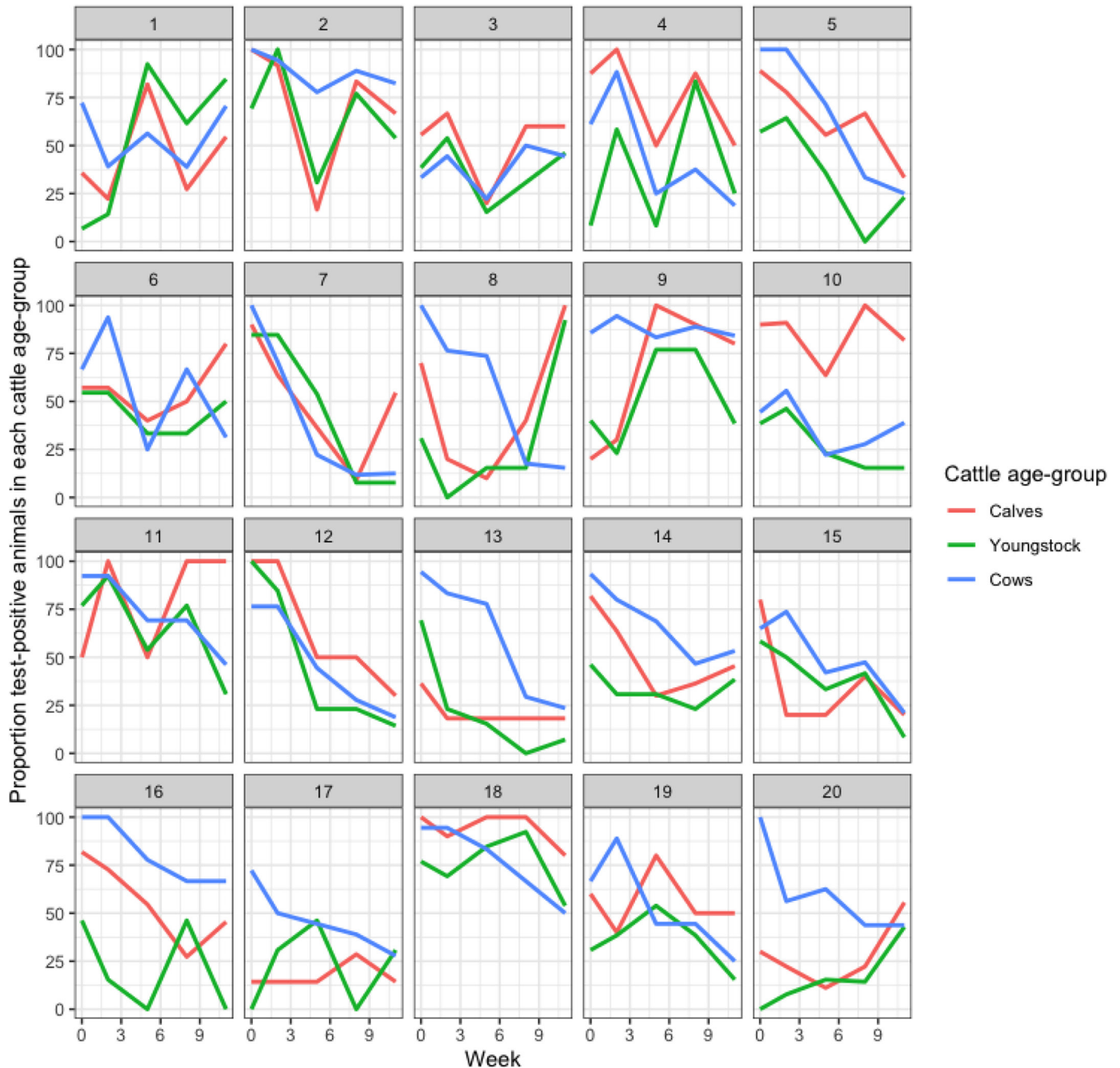


Figure 3. Epidemic trajectories for the different cohorts of cattle age groups (blue = lactating adult cows, green = youngstock, red = calves) in the 20 dairy herds in the 5 samplings during the 11-wk study period.

cows, 50% ($n = 2$) of removed youngstock and 60% ($n = 3$) of removed calves.

The percentage of cattle testing PCR-positive declined over the study period for cows and calves (Table 1). The proportion of PCR-positive cattle decreased between T_0 and T_4 from 65.1% to 31.2% (clinical cows), 66.3% to 22.2% (nonclinical cows) and 57.5% to 24.9% (calves). ELISA-positivity dropped between T_0 and T_4

for clinical cows (64.3% to 29.7%) and nonclinical cows (47.7% to 19.9%) but increased within calves (22.8% to 41.5%). Percentage PCR- and ELISA-positivity varied across the study period for youngstock (PCR min: 18.2% at T_2 , max: 33.6% at T_1 and ELISA min: 18.5% at T_4 , max: 31.8% at T_1). The proportion of milk samples that were culture-positive was low and stable in the nonclinical cow group (min: 1.9% at T_2 ,

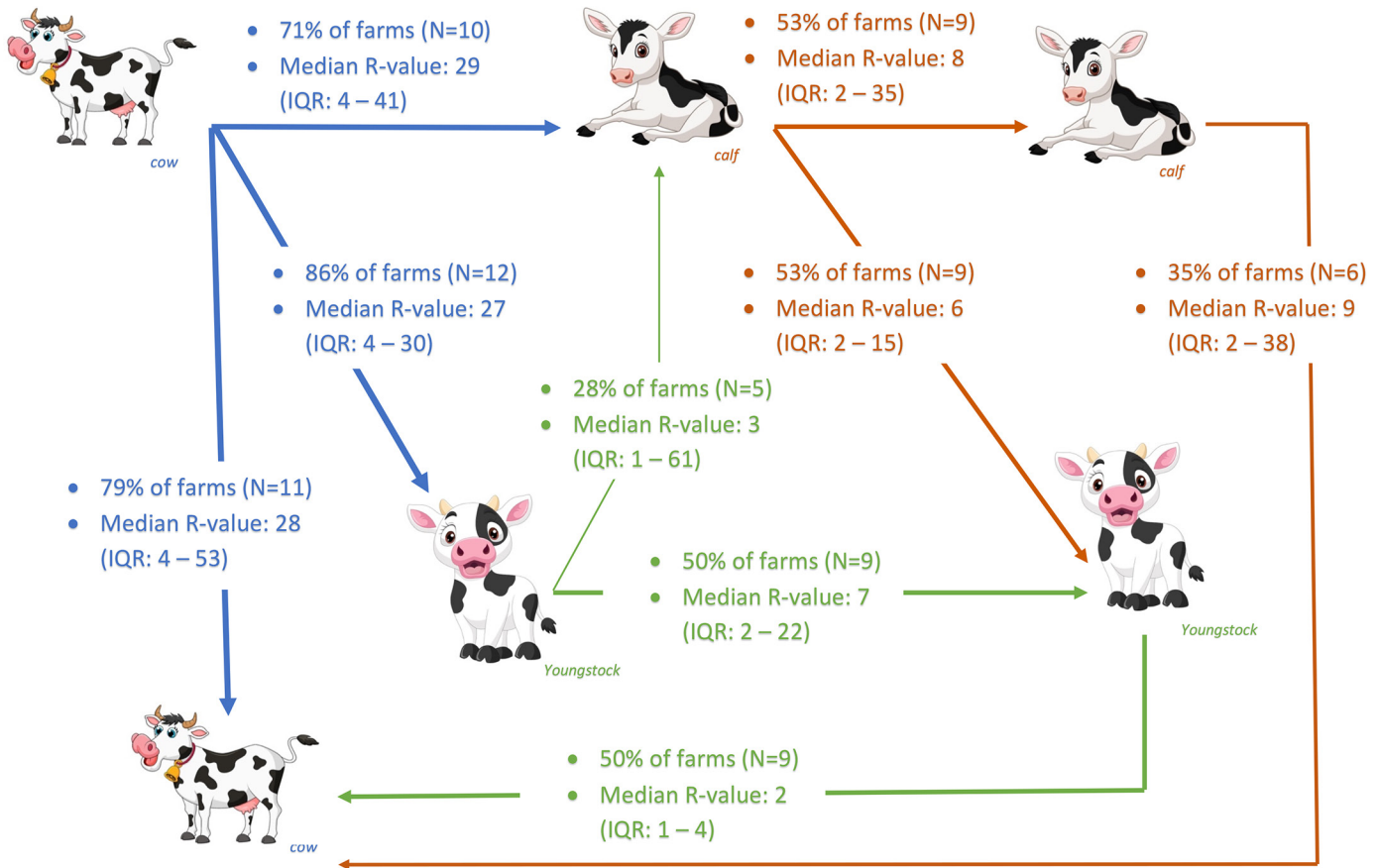


Figure 4. Within-herd transmission pathways among and from cows (blue), to and from calves (red), and to and from youngstock (green). Thickness of the lines represent the importance of a pathway, with the thickest lines indicating the most importance. IQR = interquartile range.

max: 2.7% at T_0) but declined in clinical cows from 14.5% at T_0 to 4.0% at T_1 , 4.0% at T_2 , 4.0% at T_3 and 4.1% in T_4 . A descending trend was also observed in the percentage of cattle that tested positive in 2 or more diagnostic tests (based on the number of cattle with at least 1 positive test; Table 1). The percentage decreased between T_0 and T_4 from 55.7% to 18.2% (clinical cows), 42.9% to 7.6% (nonclinical cows) and 27.7% to 13.6% (youngstock). In addition, percentage of calves fluctuated over samplings (min: 19.2% at T_4 , max: 29.1% at T_3).

Epidemic Trajectories

The epidemic trajectory for each cattle cohort group was plotted within each herd (Figure 3). These trajectories illustrate proportions of sampled cattle that tested positive in at least 1 diagnostic test, subsequently classifying them as infectious. Among the 20 herds, 18 exhibited evidence of infection having potentially spread before sampling started. Within these herds (herds 2 and 4–20), there was a descending trend in the

proportion of adult cows testing positive across sampling moments. Notably, infection dynamics in calves and youngstock fluctuated. In some herds, the number of test-positive animals within these age groups had an increasing trend (herds 1, 4, 6, 8, 9, 20; Figure 3). In 6 herds (2, 5, 7, 8, 16, and 20), all sampled cows were infected at some point in time. The same applied to calves of herds 2, 4, 8, 9, 10, 11, 12, and 18 and youngstock of herds 2 and 12.

Cow Transmission

Cow-to-cow transmission occurred in 11 herds (3, 4, 6, 9, 11, 12, 13, 14, 15, 18, and 19), with R-values ranging from 1.0 to 80, indicating that numbers of cows expected to be infected by 1 cow while infected ranged from 1 to 80 across herds (Table 2) with a median of 28.1 (Interquartile Range [IQR]: 4.3–53.1). In 3 herds (1, 10, and 17), no transmission among cows was identified. The model could not estimate parameters for 6 herds (2, 5, 7, 8, 16, and 20) because 100% of the cattle age group was infected at first sampling.

Table 1. Number (n) of cattle (clinical cows, nonclinical cows, youngstock, and calves) tested in each sampling (T), number, and percentage (%) of cattle that tested positive in PCR, ELISA, and culture, the number of cattle tested with at least one positive test outcome, and the percentage of cattle with a positive outcome that had 2 or more positive results

Item	Sampling (T)	Cattle tested (n)	PCR positive (%)	ELISA positive (%)	Culture positive (%)	At least 1 positive test (n)	Positive in ≥ 2 diagnostic tests when having 1 positive test (%)
Clinical cows	0	84	65.1	64.3	14.5	70	55.7
	1	75	63.2	57.3	4.0	60	51.7
	2	78	29.5	47.4	4.0	47	27.7
	3	77	31.2	31.2	4.0	41	24.4
	4	74	18.9	29.7	4.1	33	18.2
Nonclinical cows	0	266	66.3	47.7	2.7	210	42.9
	1	266	61.8	42.9	2.3	213	30.5
	2	263	29.7	34.2	1.9	142	21.1
	3	265	30.2	25.3	2.3	121	20.7
	4	261	22.2	19.9	2.0	105	7.6
Youngstock	0	259	33.6	25.9	NA ¹	119	27.7
	1	258	28.7	31.8	NA	121	28.9
	2	258	18.2	24.0	NA	96	13.5
	3	259	23.2	20.1	NA	97	15.5
	4	260	19.6	18.5	NA	88	13.6
Calves	0	193	57.5	22.8	NA	130	19.2
	1	189	44.4	29.6	NA	109	28.4
	2	194	26.8	28.9	NA	88	22.7
	3	195	28.7	39.5	NA	103	29.1
	4	193	24.9	41.5	NA	107	19.6

¹NA = not available.

Cow-to-youngstock and cow-to-calf transmission occurred in 12 herds (1, 4, 6, 9, 11, 12, 13, 14, 15, 17, 18, and 19), and 10 herds (4, 6, 9, 11, 12, 13, 15, 17, 18, and 19), respectively, with R-values ranging from 1.2 to

38 secondarily infected youngstock per herd (median = 26.6, IQR: 4.1–29.8) and 0.1 to 91 secondarily infected calves per herd (median = 28.9, IQR: 3.8–41.1), respectively (Table 2). Transmission from cows to all 3 cattle

Table 2. Reproduction values from cows (cow-to-calf [L-C]), cow-to-youngstock [L-Y], and cow-to-cow [L-L] transmission), youngstock (youngstock-to-calf [Y-C], youngstock-to-youngstock [Y-Y], and youngstock-to-cows [Y-L] transmission), and calves (calf-to-calf [C-C], calf-to-youngstock [C-Y], and calf-to-cow [C-L] transmission), and the infectious period of *Mycoplasma bovis* on 20 Dutch dairy farms¹

Herd	Cow transmission			Youngstock transmission			Calf transmission			Infectious period (wk)
	L-L	L-Y	L-C	Y-Y	Y-L	Y-C	C-C	C-Y	C-L	
1	0	1.2	0	21.8	0	0	0	0	3.0	23.4
2	x	x	x	x	x	x	x	x	x	20.8
3	4.3	0	0	0	0.9	0	0	0	2.1	55.0
4	80.1	29.6	91.0	0	0.1	2.6	39.6	15.0	38.2	10.3
5	x	x	x	48.0	65.0	60.5	2.0	2.9	0.1	8.0
6	53.1	38.4	41.1	0	0	1.1	3.3	0	0	12.5
7	x	x	x	4.3	4.2	4.3	1.6	0	0	5.3
8	x	x	x	2.4	0	0	0	2.1	0	10.3
9	28.4	29.4	29.9	0	4.4	0	35.1	0	0	50.7
10	0	0	0	0	0	0	8.3	2.3	0	36.1
11	28.1	25.7	21.3	95.6	107.0	75.6	13.2	14.6	14.5	20.5
12	29.8	32.8	32.8	x	x	x	x	x	x	7.0
13	4.4	4.1	3.8	0	0	0	0.5	0	0	6.5
14	1.0	6.3	0	0.7	0	0	0	0	0	12.0
15	2.7	4.0	0.1	1.6	0.4	0	59.9	41.3	47.0	5.4
16	x	x	x	0	0	0	0	0	0	15.6
17	0	4.0	2.7	0	1.7	0	0	1.1	0	6.2
18	27.8	27.5	27.9	9.1	0	0	x	x	x	31.5
19	61.2	30.1	47.9	0	1.6	0	0	6.0	0	9.6
20	x	x	x	6.8	0	0	0	9.7	0	9.9
Overall mean (median)	29 (28)	19 (27)	30 (29)	21 (7)	21 (2)	24 (3)	18 (8)	11 (6)	18 (9)	16.5

¹L = lactating adult cows >24 mo; Y = youngstock 6 to 24 mo; C = calves <6 mo. x = could not be estimated because all cattle in this age group were already *M. bovis*-positive at the first sampling.

age groups occurred in 9 herds (4, 6, 9, 11, 12, 13, 15, 18, and 19) during the study period. No transmission parameters could be estimated for any cattle age group in herd 2.

Removal rates (γ) ranged from 0.018 to 0.190, indicating the estimated mean infectious period ($1/\gamma$) for all cattle ranged from 5.3 (1/0.190) to 55.0 (1/0.018) wk across the 20 herds (Table 2). Estimated transmission and removal rates are presented in Supplemental Appendix S2 (<https://doi.org/10.6084/m9.figshare.24521008>; Biesheuvel, 2023b).

Youngstock Transmission

Youngstock-to-youngstock transmission occurred in 9 herds (1, 5, 7, 8, 11, 14, 15, 18, and 20) with R-values ranging from 0.7 to 95.6 secondarily infected youngstock per herd (median = 6.8, IQR: 2.4–21.8). No transmission within youngstock occurred in 9 herds (3, 4, 6, 9, 10, 13, 16, 17 and 19). For 2 herds (2 and 12), no transmission parameters for youngstock could be estimated. Youngstock-to-calves and youngstock-to-cow transmission occurred in 5 herds (4, 5, 6, 7, and 11) and 9 herds (3, 4, 5, 7, 9, 11, 15, 17, and 19), respectively, with R-values ranging from 1.1 to 75.6 secondarily infected calves per herd (median = 3.4, IQR: 1.1–60.5) and 0.1 to 107 secondarily infected cows per herd (median = 1.7, IQR: 0.9–4.4), respectively. Transmission from youngstock to all 3 cattle age groups occurred only in 3 herds (5, 7, and 11) within the study period.

Calf Transmission

Calf-to-calf transmission occurred in 9 herds (4, 5, 6, 7, 9, 10, 11, 13, and 15) with R-values ranging from 0.5 to 60 secondarily infected calves per herd (median = 8.3, IQR: 2–35.1; Table 2). No transmission occurred in 8 herds (1, 3, 8, 14, 16, 17, 19, and 20). For 3 herds (2, 12, and 18), the model could not calculate calf transmission parameters. Calf-to-youngstock transmission occurred in 9 herds (4, 5, 8, 10, 11, 15, 17, 19, and 20) and calf-to-cow transmission in 6 herds (1, 3, 4, 5, 11, and 15), with R-values ranging from 1.1 to 41 secondarily infected youngstock per herd (median = 6.0, IQR: 2.3–14.6) and 0.1–47 secondarily infected cows per herd (median = 8.7, IQR: 2.1–38.2), respectively. In 4 herds (4, 5, 11, and 15), transmission occurred from calves to all 3 cattle age groups within the study period.

Transmission Pathways

Given that herds were included in the study following observation of clinical signs in adult cows, hypothesized within-herd transmission pathways are illustrated,

starting from adult cows (Figure 4). In 14 herds, the model was able to calculate whether cow-to-cow, cow-to-youngstock or cow-to-calf transmission occurred. In most of these herds, transmission occurred among adult cows (11 herds, 79%; Figure 4). In 10 (71%) and 12 (86%) herds, transmission was also estimated from adult cows to calves and youngstock, respectively. Youngstock-to-calf, youngstock-to-youngstock or youngstock-to-cow transmission could be estimated for 18 herds. In 9 of those herds (50%), transmission between youngstock occurred. In 5 herds (28%) transmission from youngstock to calves could be estimated. Then, in 17 herds, calf-to-calf, calf-to-youngstock or calf-to-cow transmission could be estimated. In 9 herds (53%), transmission among calves could be estimated, and in 9 herds (53%), transmission from calves to youngstock. Finally, transmission from calves and youngstock back to adult cows occurred in 6 (35%) and 9 (50%) herds, respectively.

Risk Assessment of Farm Management

Transmission from Cows. Farms with cow-to-cow transmission were more likely to have clean and dry sick cow pens ($P < 0.01$). Additionally, these farms tended to have sick cow pens with an area of at least 10 m² ($P = 0.10$, 95% CI: 0.42–1292.0). Furthermore, relative to the number of cows present on the farm, these farms tended to have fewer water troughs and feeding stations ($P = 0.10$, 95% CI: 0.77–1.02; $P = 0.13$, 95% CI: 0.77–1.04) compared with farms without cow-to-cow transmission (Table 3). No differences in farm management factors were identified between farms with and without cow-to-calf transmission and cow-to-youngstock transmission.

Transmission from Youngstock. Farms with youngstock-to-youngstock transmission tended to have separate caretakers more frequently for youngstock >6 mo ($P = 0.08$) compared with farms without youngstock-to-youngstock transmission (Table 3).

In the case of farms with youngstock-to-calf transmission, 2 management factors were notable. First, these farms were more likely to hire a contractor for labor ($P = 0.04$, 95% CI: 0.01–1.61). Second, they might have less frequent employment of caretakers for calves <6 mo ($P = 0.12$, 95% CI: 0.62–99.9; Table 3).

No differences in farm management factors were identified between farms with and without youngstock-to-cow transmission.

Transmission from Calves. Farms with calf-to-calf transmission tended to hire a contractor for labor more frequently ($P = 0.08$). Additionally, they might have external employees ($P = 0.15$, 95% CI: 0.52–87.93), suboptimal calf housing ventilation ($P = 0.13$, 95% CI: 0.00–1.78) and >3 individuals involved in animal care

Table 3. Number, percentage, mean (SD), and corresponding *P*-values of the associated farm management factors ($P < 0.15$) for each of the transmission pathways using Fisher's exact test for categorical variables and Poisson regression for continuous variables

Transmission pathway, farm factor ¹	No transmission, n (%)	Transmission, n (%)	All farms, n (%)	<i>P</i> -value	95% CI	
					Lower	Upper
L-L						
Clean and dry sick cow pen	0 (0.0)	11 (100)	11 (78.6)	<0.01	NA ²	NA
Sick cow pens at least 10 m ²	1 (33.3)	10 (90.9)	11 (78.6)	0.10	0.42	1292.0
Y-Y						
Separate caretakers for calves >6 mo	0 (0)	4 (44.4)	4 (22.2)	0.08	NA	NA
Y-C						
Separate caretakers for calves <6 mo	9 (69.2)	1 (20.0)	10 (55.6)	0.12	0.62	99.9
Hiring contractor for labor	1 (7.7)	3 (60.0)	4 (22.2)	0.04	0.01	1.61
C-C						
Hiring contractor for labor	0 (0)	4 (44.4)	4 (23.5)	0.08	NA	NA
External employee(s)	2 (25.0)	6 (66.7)	8 (47.1)	0.15	0.52	87.93
Suboptimal calf housing ventilation	1 (12.5)	5 (55.6)	6 (35.3)	0.13	0.00	1.78
>3 people involved in animal care	3 (37.5)	7 (77.8)	10 (58.8)	0.15	0.49	86.29
C-L						
External employee(s)	7 (63.6)	1 (16.7)	8 (47.1)	0.13	0.00	1.78
C-Y						
Clean boots	5 (62.5)	9 (100)	14 (82.4)	0.08	NA	NA
Recent stressful situation	1 (12.5)	5 (55.6)	6 (35.3)	0.13	0.56	471.48
All farms (Poisson regression)						
L-L, mean (SD)						
Number of water troughs	7.3 (1.2)	6.8 (5.4)	7.3 (4.5)	0.10	0.77	1.02
Number of feeding stations	2.0 (3.5)	3.6 (4.4)	3.8 (3.6)	0.13	0.77	1.04

¹L = lactating adult cows >24 mo; Y = youngstock 6 to 24 mo; C = calves <6 mo.²NA = exact confidence intervals are not possible with zero count cells.

($P = 0.15$, 95% CI: 0.49–86.29) compared with farms without calf-to-calf transmission (Table 3).

Farms with calf-to-youngstock transmission tended to be more prone to maintaining clean boots ($P = 0.08$) and might have recently experienced a stressful situation such as claw trimming or clipping ($P = 0.13$, 95% CI: 0.56–471.48) compared with farms without calf-to-youngstock transmission (Table 3).

Farms with calf-to-cow transmission might less often employ an external employee ($P = 0.13$, 95% CI: 0.00–1.78) compared with farms without calf-to-cow transmission (Table 3).

DISCUSSION

Likely *M. bovis* transmission pathways were identified from longitudinal data collected in 20 clinically *M. bovis*-infected dairy herds in the Netherlands. Among the 14 herds where not all cows were *M. bovis*-positive during initial sampling, transmission primarily occurred from adult cows to youngstock (12 herds), calves (10 herds) and other adult cows (11 herds). Among the 17 herds where not all calves were *M. bovis*-positive during the initial sampling, calves were responsible for infecting other calves and youngstock in 9 and 9 herds, respectively. Finally, in the 18 herds where not all youngstock were *M. bovis*-positive during the initial sampling, youngstock infected other youngstock and

adult cows in 9 and 9 herds, respectively. Transmission from calves to adult cows and from youngstock to calves only occurred in 6 and 5 herds, respectively. It is not surprising that most herds experienced transmission from cows to the other cattle age groups because the presence of clinical signs in adult cows was a selection criterion. Consequently, estimated transmission parameters and pathways were applicable solely to herds that experienced an outbreak commencing in this specific age group.

Furthermore, in most herds, the study started ~2 wk after the first observed clinical signs of *M. bovis* infection. Although this timeline is rapid from a project management perspective, it appeared to be inadequate for capturing the complete on-farm *M. bovis* epidemic (Figure 4). The number of infected cows decreased over time across almost all herds. Also, in some herds, all sampled cattle were already positive at T_0 (Penterman et al., 2022). Estimated transmission parameters substantiated this observation, revealing a wide range of mean infectious periods for transmission ranging from 5.4 to 55 wk across the 20 herds. The basic reproduction ratios (representing the average number of secondary infections caused by 1 infectious animal over the entire course of its infectious period in an otherwise fully susceptible population; Diekmann et al., 1990) ranged from 1.0 to 80 for cow-to-cow transmission, indicating *M. bovis* spreads very fast in certain herds.

Moreover, employing a whole-herd sampling approach rather than subsampling a group of cattle might have addressed the issue of 100% positivity at T_0 . However, this would have been very expensive. The wide range of estimated infectious periods, with a maximum of 55 wk in the 20 herds, could reflect the chronic nature of *M. bovis* infections within a herd.

Nonetheless, the data used have limitations regarding sensitivity, specificity, and sampling time. Limitations regarding sensitivity and specificity will be discussed further in this section. Moreover, the relative simplicity of the SIR model may have overestimated the infectious period. As such, additional studies are needed to verify whether these extended infectious periods truly reflect herds' chronicity. Furthermore, the SIR model was based on the assumptions of a closed herd, with no introduction of new cattle into the herd during the study period and thus no additional external introduction of *M. bovis* beyond the existing outbreak, and no between-group movement of cattle. Although neither assumption was verified, given the study's 3-mo duration, they were deemed reasonable. Additionally, most cattle were sampled 4 to 5 times, with only a small number lost for follow-up.

Diagnosing *M. bovis* is challenging. The sensitivity of the Bio-X K260 ELISA, designed to detect seroconversion of cattle exposed to *M. bovis*, was relatively low at 14.1%, likely causing a high proportion of false-negatives. Conversely, the Bio-X K260 ELISA demonstrated a high specificity of 99%, which should result in very few false-positives. Typically, ELISAs are employed to detect antibodies against past infections; however, the Bio-X K-302 ELISA, a similar ELISA designed to detect seropositive animals, was described as being able to detect clinical cattle (Petersen et al., 2020). Similar trends were observed for the Bio-X K260 ELISA that exhibited enhanced sensitivity (from 14.1 to 70.7%; BCI: 63.7–77.6%) when used in clinical herds experiencing a recent outbreak (Veldhuis et al., 2023). Notably, the Bio-X K302 ELISA failed to detect antibodies in calves <3 mo exposed to *M. bovis* (Petersen et al., 2018b), although its sensitivity was slightly better (Schibrowski et al., 2018). This suggests a potential for a higher proportion false-negatives among calves < 6 mo in the ELISA, assuming the Bio-X K260 ELISA performs similarly in young calves. However, Sachse et al. (2010) reported successful detection of *M. bovis* in conjunctival swabs using PCR, particularly in calves displaying respiratory symptoms.

Similar diagnostic challenges apply to adult cows, where, for instance, milk samples are more likely to detect *M. bovis* mastitis rather than systemic disease. Thus, detecting cows with arthritis or other symptoms caused by *M. bovis* would probably be challenging

when only using milk samples (Petersen et al., 2020). Interpreting test results of ELISA, PCR, and culture in parallel when categorizing each sampled animal as S, I or R at each sampling increases sensitivity but reduces specificity compared with using each diagnostic method individually. Whereas the ELISA used exhibited a low sensitivity (14.1%) and a single milk culture displayed a sensitivity of 25% (González and Wilson, 2002), specificity was reported to be consistently high for culture, PCR and ELISA (>95%). Consequently, the focus in this study was to increase sensitivity, even if it meant compromising specificity and potentially increasing the number of false-positive cases. Notably, in the plotted epidemic trajectories per herd, certain cattle displayed a sequence of positive-negative-positive test outcomes across 3 consecutive rounds of testing (Figure 3). From a biological perspective, it seems very unlikely that these results reflected genuine antibody titers, given that titers generally decline approximately 8 to 9 wk after the onset of clinical signs (Byrne et al., 2000; Petersen et al., 2018a). To address this, a sensitivity analysis was performed, adjusting the data in 3 ways: (1) altering a sequence of positive-negative-positive outcomes to positive-positive-positive; (2) modifying the ELISA cut-off value; and (3) combining both adjustments. Whereas some herd's fitted epidemic trajectories seemed to improve, particularly under Scenario 3, no solid conclusions could be drawn from adjusted data scenarios. Additionally, no major changes in on-farm transmission pathways were observed. Consequently, the decision was made to present uncorrected results.

A future recommendation would be utilization a Bayesian latent class model that could incorporate test sensitivity and specificity. This model could also use quantitative diagnostic test results from tests such as ELISA and PCR, allowing to determine whether the existing results align or if accuracy could be improved. Such a model would enable exploration of the influence of individual animal characteristics (e.g., clinical status) on transmission dynamics. Regrettably, these types of models are not currently well-established and thus not readily accessible for implementation.

The standard procedure outlined by the National Mastitis Council (NMC; Middleton et al., 2017) involves culturing *Mycoplasma* with the addition of 10% CO₂, a step not used in this study, where cultures were developed under regular ambient air conditions. However, it is important to note that NMC's recommendation lack empirical derivation (Lowe et al., 2018). Moreover, recent preliminary findings from a University of Calgary experiment involving 17 isolates cultured in triplicate under ambient air, 5% and 10% CO₂, plus 3 dilutions, supported these observations. Detection rates exhibited variation on d 3 and 5 of incubation, with

lower rates for isolates cultured under ambient air. This difference on d 5 was specific to the 10^{-6} dilution range, and not affecting 10^{-4} and 10^{-5} dilutions. However, no distinctions were discerned after 7 or 10 d of incubation, suggesting *Mycoplasma* has similar detection rates across ambient air, 5% and 10% CO₂ on d 7 and 10 of incubation, with the latter being the incubation duration recommended by the NMC and used in our study. As such, we are confident in concluding that culturing under ambient conditions did not have an effect on the sensitivity of the culture method.

Estimated transmission parameters and pathways varied among herds. Some herds demonstrated less intense transmission, characterized by lower transmission rates and longer infectious periods, whereas others had rapid dissemination of *M. bovis* infection across all age groups. The initial risk assessment conducted at the onset of the study provided insights into potential farm factors that might explain this heterogeneity. Farms where transmission occurred among cows tended to have a smaller ratio of water troughs and feeding stations to the total number of cows present compared with farms without this specific transmission pathway. Notably, a study utilizing data from the same 20 herds indicated the presence of *M. bovis* in water troughs (Penterman et al., 2022). *Mycoplasma bovis* has the capability to persist in sand for months, particularly under cool and moist conditions (Justice-Allen et al., 2010), suggesting the possibility of transmission through indirect contacts such as drinking troughs and feeding stations. Furthermore, these farms more frequently maintained clean and dry sick cow pens that were at least 10 m². It is plausible that farms with transmission between cows might be more inclined to take precautions to prevent *M. bovis* from spreading further and be more aware of the need to keep sick cow pens clean and dry.

Farms where transmission occurred from calf-to-calf displayed certain characteristics, such as hiring contractors for labor, more frequent utilization of external employees, more people involved in animal care, and suboptimal ventilation in calf housing. These findings aligned with the established understanding that ventilation, air quality and shared air spaces are associated with respiratory diseases in calves more broadly (Butler et al., 2000). Contractors, which were also associated with transmission from youngstock to calves, and external employees, associated with transmission from calves to cows, are known risk factors for introducing *Mycoplasma* into a herd due to potential biosecurity risks (Haapala et al., 2021). The number of people engaged in animal care is a good proxy for herd size (Frössling and Nöremark, 2016), and increasing herd size is an important risk factor for *M. bovis* transmission (Fox et al., 2003; Nicholas et al., 2016). In contrast,

farms where transmission occurred from youngstock to youngstock tended to have designated caretakers more frequently for youngstock >6 mo, thereby contributing to increased transmission within that specific age group while limiting transmission to other age groups. Conversely, farms with transmission from youngstock to calves more commonly employed the same caretaker for calves < 6 mo, promoting transmission between youngstock and calves. Although previous studies have predominantly focused on introducing *Mycoplasma* infections into the herd through cattle purchases (Schibrowski et al., 2018; Murai and Higuchi, 2019) or biosecurity risks arising from human visitors or veterinarians entering the barn (Haapala et al., 2021), the role of human-animal contact concerning the on-farm transmission of *Mycoplasma*, particularly across age groups, remains relatively underexplored.

Due to the relatively small sample size of only 20 farms, it was impossible to make more complex statistical inferences about associations. Therefore, findings presented in this study are exploratory and potentially relevant factors could have been missed. Subsequent research is needed to establish more robust relationships between estimated transmission pathways and the risk factors identified. Moreover, factors such as differences in *M. bovis* strain-to-strain virulence and individual susceptibility could have a role. Furthermore, it is important to acknowledge that biases can emerge when employing questionnaires in a study. Information bias, for instance, can manifest due to several factors, including questions that permit subjectivity in interpretation by different individuals administering the questionnaire. An approach to mitigate this bias involves using clear, unambiguous questions, and minimizing the number of questioners. If multiple questioners are involved, ensuring consistency in question interpretation and answer collection through appropriate training can curb differences. In this study information bias was limited as much as possible. Only 2 experienced technicians, well-versed in udder health, conducted the questionnaires on the 20 farms. However, if information bias did surface, it was expected to be nondifferential and would have led to underestimation of the effect of the risk factors.

Initially, a susceptible, exposed, infected, and removed model was considered. However, due to a lack of comprehensive knowledge into the latency period specific to *M. bovis* and its potential variability among cows, youngstock, and calves, a more simplified SIR model was adopted.

It is important to highlight that, to the best of the authors' knowledge, this is the first study calculating transmission parameters and pathways of *M. bovis* within dairy herds. Despite its inherent limitations,

results obtained from this study have much potential for enhancing our comprehension of *M. bovis* on-farm transmission dynamics within dairy herds. We firmly believe that these findings could serve as valuable evidence for informing development and implementation of effective control strategies to manage *M. bovis* transmission.

CONCLUSIONS

On dairy farms with a clinical outbreak of *M. bovis* where the nidus of infection was initially found in adult cows, the dynamics of transmission across different cattle age groups was highly variable among herds. Transmission pathways encompassing adult cows, youngstock, and calves displayed markedly diverse transmission rates, with the highest rates observed in pathways involving cows transmitting to other cows, calves and youngstock. Of notable significance were the cow-to-cow, cow-to-calf and cow-to-youngstock pathways, followed by calf-to-calf and calf-to-youngstock, youngstock-to-youngstock and youngstock-to-cows pathways. While the study's focus centered on an outbreak of *M. bovis* in adult dairy cows, making it more likely that transmission rates are higher for pathways from cows, it is imperative not to disregard the effect of transmission to and among youngstock and calves. The pathways significantly contribute to the on-farm outbreak, underscoring the necessity to recognize their role. The heterogeneity observed among farms in terms of transmission parameters and pathways could potentially be related to factors encompassing internal biosecurity practices (such as distinct caretakers for various age groups and number of people involved), external biosecurity measures (involvement of contractors and external employees) or even indirect transmission routes (such as the number of feed and water stations).

ACKNOWLEDGMENTS

This study was funded by ZuivelNL and supported by the Industrial Research Chair in Infectious Diseases of Dairy Cattle, funded by Canada's Natural Sciences and Engineering Research Council Industrial Research Chair Program (Ottawa, ON, Canada), with industry contributions from Alberta Milk (Edmonton, AB, Canada), the Dairy Farmers of Canada (Ottawa, ON, Canada), Westgen Endowment Fund (Milner, BC, Canada), the BC Dairy Association (Burnaby, BC, Canada), Canadian Dairy Network (Guelph, ON, Canada), CanWest DHI (Guelph, ON, Canada), SaskMilk (Regina, SK, Canada), Dairy Farmers of Manitoba (Winnipeg, MB, Canada), and MSD Animal Health (Kirkland, QC, Canada). Marit Biesheuvel is also partly funded






through her Alberta Graduate Excellence Scholarship–International by the Alberta Government. The authors have not stated any conflicts of interest.

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