***Mycoplasma bovis* and other *Mollicutes* in replacement dairy heifers from *Mycoplasma bovis*-infected and uninfected herds: A 2-year longitudinal study**

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ABSTRACT

Replacement dairy heifers exposed to *Mycoplasma bovis* as calves may be at risk of future clinical disease and pathogen transmission, both within and between herds; however, little information is available about these risks. We conducted a 2-yr longitudinal (panel) study starting with 450 heifer calves reared to weaning in 8 herds (7 *M. bovis* infected with clinical disease, 1 uninfected) under the same ownership. After weaning, heifers were commingled and managed with non-study heifers at a single heifer rearing facility. Nose, conjunctival, and vaginal swabs were collected along with a blood sample at weaning, prebreeding, precalving, and approximately 1 mo postcalving. Additionally, a colostrum sample was collected upon calving and a composite milk sample was collected 1 mo postcalving. The swabs, colostrum, and milk samples were cultured for *Mycoplasma* spp., and serum from the blood was evaluated for serological evidence of exposure to *M. bovis* using an ELISA. Despite a high *M. bovis* ELISA seroprevalence at weaning in the heifers from the 7 *M. bovis*-infected herds with clinical disease [72% (289/400); range by herd: 28–98%], *M. bovis* was isolated from only 4% (16/400) of the same heifers at the same time. In heifers from the uninfected herd at weaning, *M. bovis* seroprevalence was 2% (1/50) and *M. bovis* was not detected by culture. *Mycoplasma bovis* was isolated from 0.5% (2/414) of heifers at prebreeding, 0% (0/374) of heifers at precalving, and 0.3% (1/356) of heifers 1 mo postcalving. The nose was the predominant anatomical site of *M. bovis* colonization (74%; 14/19 culture positives). A single heifer (from an *M. bovis*-infected herd with clinical disease) was repeatedly detected with *M. bovis* in its nose at weaning, prebreeding, and postcalv-

ing samplings. This demonstrates the possibility, albeit rare, of a long-term *M. bovis* carrier state in replacement heifers exposed to *M. bovis* as calves, up to at least 1 mo after entry into the milking herd. No *M. bovis* clinical disease was detected in any heifer from weaning through to the end of the study (approximately 1 mo after calving). *Acholeplasma* spp. were commonly isolated throughout the study. *Mycoplasma bovis*, *Mycoplasma bovoculi*, and *Mycoplasma bovirhinis* were isolated infrequently. *Mycoplasma bovis* seroprevalences at prebreeding, precalving, and postcalving samplings were 27% (112/414), 12% (46/374), and 18% (65/356), respectively. Overall, the results show that replacement heifers from groups exposed to *M. bovis* preweaning can become colonized with *M. bovis* and that colonization can, uncommonly, be present after their first calving. For groups of 50 or more heifers exposed to *M. bovis* preweaning, there is at least a nontrivial probability that the group will contain at least 1 shedding heifer postcalving.

Key words: *Mycoplasma bovis*, replacement heifer, subclinical, colonization

INTRODUCTION

Mycoplasma bovis is recognized worldwide as a pathogen of economic and biosecurity importance due to its capacity to cause severe mastitis, arthritis, and pneumonia in adult cattle. Young calves may become infected after drinking *M. bovis*-contaminated milk and present with clinical manifestations including otitis media, pneumonia, and arthritis (Maunsell and Donovan, 2009). *Mycoplasma bovis* prevalence and transmission has been studied extensively in preweaned calves (Stipkovits et al., 2000; Maunsell and Donovan, 2009; Maunsell et al., 2012), but few studies have examined *M. bovis* prevalence in replacement heifers between weaning and their entry into the milking herd. Bennett and Jasper (1977) studied apparent prevalences of *My-*

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coplasma in the nose and *Mycoplasma* seroprevalences repeatedly over an 8-mo period in approximately 200 heifers from 6 different age groups between 0 and 24 mo of age, from both *Mycoplasma* mastitis and non-*Mycoplasma* mastitis herds. They observed high apparent total prevalences (30.0–45.5% for different age groups) and concluded that *M. bovis* nasal prevalence is higher in heifers from the *Mycoplasma* mastitis herds up to 1 yr of age. Pfützner and Sachse (1996) stated that clinically healthy calves and young cattle can harbor *M. bovis* in their respiratory tract for months to years but provided no supporting evidence.

Replacement heifers are frequently sourced when a dairy herd is expanding. Understanding the prevalence of *M. bovis* in exposed replacement heifers over time is necessary for risk assessment of replacement heifer purchases at any age. Evaluating *M. bovis* prevalence at different anatomical sites is necessary to inform the design of sampling protocols for replacement heifers prepurchase to detect *M. bovis*-infected animals. Serology may also be a useful tool for detecting *M. bovis* carriers or higher risk replacement heifers that have been previously exposed to *M. bovis*. However, Petersen et al. (2018) suggested that *M. bovis* antibody responses are short lived in naturally exposed and diseased cows. Additionally, no studies describe the persistence of *M. bovis* antibodies in replacement heifers exposed to *M. bovis* as calves. Although *M. bovis* is the major *Mycoplasma* of pathogenic concern in younger cattle, other mycoplasmas can also cause disease and commensal mycoplasmas can be isolated in mixed infections with other known pathogens (Maunsell and Donovan, 2009).

For replacement heifers potentially exposed to *M. bovis* as calves in herds with *M. bovis* clinical disease in the milking group, the objectives of this study were to (1) describe the apparent prevalences of *Mollicutes* on the mucosal surface of the nose, conjunctiva, and vagina of such heifers at weaning, prebreeding, precalving, and postcalving and in colostrum upon calving and milk postcalving; (2) describe *M. bovis* ELISA seroprevalences and persistence of antibody responses of the same heifers at weaning, prebreeding, precalving, and postcalving; (3) compare weaning results from objectives 1 and 2 with those in replacement heifers from an uninfected herd; and (4) estimate probabilities of *M. bovis* shedding in heifer groups born and raised in herds with *M. bovis* clinical disease in the milking group.

MATERIALS AND METHODS

Study Overview

A prospective longitudinal (panel) study was conducted using 450 heifers born in 2013 from 8 dairy herds

under common ownership. Prior to weaning of the heifers, 7 herds had diagnosed *M. bovis* clinical disease in the milking group via samples collected from clinically affected cattle and tested with PCR and culture. In at least 3 of these herds, *M. bovis* clinical disease also occurred in preweaned calves. In the eighth herd, no suspected or diagnosed *M. bovis* clinical disease cases had ever been detected in either the milking group before heifer calf weaning or preweaned calves. All heifer calf groups were fed hospital group milk (i.e., milk from cows removed from the main milking group, mostly lame or mastitic cows receiving treatment) along with fresh milk from the main milking group. No milk pasteurizers or milk treatments (e.g., acidifiers) were used in any of the herds. Heifers were enrolled in the study at weaning. Upon weaning, all study heifers were moved immediately to a single heifer rearing grazing area. Study heifers were commingled with study heifers from other herds and non-study heifers under the same ownership and were grazed in smaller subgroups that varied in size according to paddock size and pasture availability. Heifers were initially managed in groups according to their season of birth. However, groups were subsequently restructured based on management decisions regarding heifer BW, pregnancy status, and destination herd, and heifers were therefore commingled with other study and non-study heifers (some from other seasons of birth). Approximately 1 mo before calving, heifers were returned to their herd of origin or a different herd (including some non-study herds under the same ownership). Heifers were assessed for evidence of *M. bovis* colonization and for serological evidence of exposure to *M. bovis* at weaning, prebreeding, precalving, calving (colostrum only), and postcalving.

Description of Herd *M. bovis* Clinical Patterns

Three herds (herds 1, 4, and 5) calved only in autumn, 3 herds (herd 6, 7, and 8) calved only in spring, and 2 herds (herds 2 and 3) practiced autumn and spring calving (split calving). Herds 1 through 7 had clinical *M. bovis* disease (herds 1–6: multiple cases, both mastitis cases and arthritis cases; herd 7: only 1 case, a cow with arthritis) in the milking group at some time during the period when the study heifers born in those herds were fed milk. Herd 1 also had clinical arthritis *M. bovis* cases in rising 2-yr-old heifers precalving during this period. No clinical cases consistent with *M. bovis* disease were detected in herd 8.

A commercially available PCR (Thermo Scientific Pathoproof Major 4.2, Dairy Technical Services, Melbourne, VIC, Australia) was used to test samples from most clinically affected cattle and describe each herd's clinical history in relation to *M. bovis*. The PCR results

Table 1. Study milking group size, season of birth of study calves, and number of clinical *Mycoplasma bovis* cases in the dry cows and main milking herd as well as timing of occurrence of confirmed and suspected *M. bovis* clinical cases in relation to the herd's weaning sample date (d 0) for the study calves

Herd no.	Milking group size ¹	Season of birth	Range of calf births before weaning (d)	Total no. of confirmed and suspected <i>M. bovis</i> cases ²	Timing of occurrence (d) ³			
					< -150	-150 to -101	-100 to -51	-50 to 0
1	880	Autumn	-153 to -106	84	23 ⁴	24	34	3
2	1,612	Autumn	-142 to -90	57	0	0	41	16
2	1,612	Spring	-136 to -101	94	36	21	37	0
3	1,354	Autumn	-182 to -118	9	0	3	6	0
4	958	Autumn	-149 to -79	21	16 ⁴	0	2	3
5	946	Autumn	-147 to -103	6	0	0	0	6
6	274	Spring	-134 to -106	21	0	0	0	21
7	1,055	Spring	-133 to -90	1	0	0	0	1
8	337	Spring	-121 to -86	0	0	0	0	0

¹Approximate number of cows. Milking group size as recorded closest to d 0 (main milking group and hospital group combined).

²Numbers of cases from the previous milking season through the herd's weaning sample date. Herds 1 to 6 had both clinical mastitis cases and arthritis cases; in herd 7, the 1 case had arthritis.

³Time of occurrence was variously based on dates of diagnosis, culling, death, or euthanasia.

⁴All clinical cases for this period were diagnosed before the period when study heifer calves born in those herds were fed milk from cows in that herd.

from this laboratory were reported as not detected or were classified based on a cycle threshold (CT) value as high (CT <22), medium (CT 22–30), low (CT 30.1–37), or caution (CT 37.1–40). One sample was tested with culture at the local government laboratory (Animal Health Laboratory, Prospect, Tasmania, Australia).

Table 1 summarizes approximate herd sizes, season in which study calves were born (autumn or spring), historical number of *M. bovis* cases in each herd, and timing of their diagnosis, culling, death, or euthanasia in relation to the herd's weaning sample date (d 0).

In herd 1, 23 clinical mastitis *M. bovis* cases were identified from d -313 during lactations that commenced in the previous calving period. During the calving period when the study calves were born, between d -107 and -22, a further 61 cases (22 arthritis, 35 mastitis, and 4 with both mastitis and arthritis) were identified. These cases began with arthritis or tenosynovitis in several heifers precalving and continued with cases of mastitis in the milking cows, with and without concurrent arthritis. Of the total 84 *M. bovis* cases, 52 were confirmed with PCR and the remaining 32 had clinical signs consistent with previously diagnosed cases in this herd. Bulk tank milk was *M. bovis* PCR positive on d -363, -340, -329, -321, and 50.

In herd 2, for autumn-born calves, the first clinical mastitis *M. bovis* cases were diagnosed at d -83. A total of 57 clinical mastitis *M. bovis* cases were diagnosed before weaning. Bulk tank milk was *M. bovis* PCR positive on d -19 and d 104. For spring-born calves, the first cases of *M. bovis* clinical mastitis were diagnosed at d -174. A total of 94 clinical mastitis *M. bovis* cases (82 confirmed with PCR; 12 with consistent clinical

signs) were diagnosed before weaning (d 0). Of those 94 clinical mastitis *M. bovis* cases, 51 were after the birth of the first study heifer (d -136), occurring between d -135 and -76. Bulk tank milk was *M. bovis* PCR positive on d -110 and d 13.

In herd 3, 8 clinical *M. bovis* mastitis cases were detected via PCR or culture between d -125 and -56. One clinical arthritis *M. bovis* case was detected via PCR on d -110. Bulk tank milk was *M. bovis* PCR positive on d -53.

In herd 4, 16 clinical mastitis case were *M. bovis* PCR positive on d -305, during lactations that commenced in the previous calving period. During the calving period when the study calves were born, 5 cases of clinical mastitis were *M. bovis* PCR positive, on d -52, -45, and -39. Bulk tank milk was *M. bovis* PCR positive on d -360, -336, -314, and -273.

In herd 5, 5 clinical mastitis cases were *M. bovis* PCR positive at d -49, and 1 additional clinical mastitis case was PCR positive on d -26. Bulk tank milk was *M. bovis* PCR positive on d -277.

In herd 6, 21 cases of clinical mastitis were *M. bovis* PCR positive between d -39 and -25. Bulk tank milk was *M. bovis* PCR positive on d -78, -43, and -16.

In herd 7, 1 case of *M. bovis* arthritis was detected via culture on d -14. Bulk tank milk was *M. bovis* PCR positive on d -76 and *M. bovis* PCR negative on d -40 and -14.

In herd 8, no *M. bovis* clinical disease had ever been detected. The bulk tank milk had an *M. bovis* PCR caution result (CT 38.8) on d -77 and was PCR negative on d -41 and -15. Extensive testing was conducted on milk samples from clinical mastitis cases from 5 wk

before to 2 mo after the bulk tank milk *M. bovis* PCR caution result, and no *Mycoplasma* culture positive cases were detected.

Heifer Selection and Sampling

A total of 250 autumn-born heifers and 200 spring-born heifers were systematically selected (e.g., approximately every fifth heifer calf from a group of 250 calves) from 8 herds. This included 50 from either season from each of 7 herds (herds 1 and 3–8), and 50 autumn-born (March–June 2013) and 50 spring-born (July–August 2013) heifers from the split-calving herd (herd 2). Each heifer was sampled on 5 separate occasions: weaning (August, September, or November 2013), prebreeding (May or September 2014), precalving (February or June 2015), calving (March, April, July, August, or September 2015), and approximately 1 mo (21–65 d) postcalving (April, May, August, September, or November 2015). Within each herd (and within each calving group in herd 2), all study calves were weaned and sampled on the same day. Most heifers (339/450) were weaned between 79 and 153 d of age, with a few (39/450; all from herd 3) weaned between 154 and 182 d. Birth dates were not available for 72 heifers. Within each herd, regardless of age at weaning, the same management practices were applied to all heifer calves until weaning.

Swabs and blood were collected by the study investigators from each heifer at weaning, prebreeding, precalving, and postcalving. Swab (15-cm rayon tip contained in Amies transport medium; FL Medical, Torreglia, Italy) samples included the right or left nostril (nose), the right or left conjunctival sac, and the vagina. Blood samples were collected from the tail vein. In addition, a composite milk sample was collected from each heifer postcalving. Timing of postcalving sampling was approximately 1 mo after calving to allow enough time for potential seroconversion of any heifers first exposed to *M. bovis* soon after entry into the milking herd. A composite colostrum sample was also collected from approximately two-thirds of the heifers at calving by farm workers. Some heifers were not available at some samplings after weaning due to incomplete mustering, death, culling, or failure to conceive. Some autumn-born heifers that did not reach minimum breeding BW at the time their prebreeding sampling was due were instead held over and sampled and bred with the spring-born heifers prebreeding and thereafter. Similarly, some spring-born heifers that did not reach minimum breeding BW at the time their prebreeding sampling was due were not eligible for subsequent sampling as this would have been outside the time frame of the study (2 yr). Of the 374 heifers sampled precalving, 47.3% (n = 177)

were returned to their original herd, 15.0% (n = 56) were returned to a different study herd with a history of *M. bovis* clinical disease, and 37.7% (n = 141) were sent to a non-study herd.

Sample and Laboratory Analyses

Mycoplasma Species Isolation and Identification. Swab, colostrum, and milk samples were cultured on *Mycoplasma* agar [*Mycoplasma* agar base (Oxoid CM0401, Waltham, MA); Milli-Q water (Millipore, Molsheim, France); 0.2% wt/vol calf thymus DNA (Sigma D1501, Sigma, St. Louis, MO); *Mycoplasma* Selective Supplement G (Oxoid SR0059C); prepared by Elizabeth Macarthur Agricultural Institute, NSW Department of Primary Industries, NSW, Australia]. Agar plates were incubated at 37°C in candle jars for 5 to 10 d. Following incubation, the plates were examined for colonies with gross morphology consistent with *Mycoplasma* species. For each culture, several colonies were selected and aseptically transferred into 40 µL of sterile PBS. The PBS suspension was vortexed, with 10 µL subcultured onto *Mycoplasma* agar and 5 µL used in each PCR reaction. Colonies from the subculture plates were subsequently harvested and stored in Cryobead tubes (Protect Multipurpose, Thermo Fisher Scientific, Waltham, MA) at –80°C.

PCR. Two PCR assays were used to determine the identity of the culture isolates. First, isolates were processed using a *Mycoplasma* spp. conventional PCR as described by Parker et al. (2017b; Table 2) and adapted from Tang et al. (2000). All amplified PCR products underwent Sanger sequencing at the Australian Genome Research Facility Ltd. (Westmead, NSW, Australia) to validate species identification. Isolates that did not amplify in the *Mycoplasma* spp. conventional PCR were processed using an *Acholeplasma* spp. conventional PCR designed using the Primer3 software program (Untergasser et al., 2012; Table 2). Isolates from the *Acholeplasma* spp. conventional PCR did not undergo Sanger sequencing. For each PCR assay, reaction mixtures contained 0.25 mM dNTPs, 2.5 mM MgCl₂, 1.5 U of GoTaq (Promega Corporation, Madison, WI), 0.25 µM each primer, 8 µL of 5× buffer, and 5 µL of PBS cell suspension in a final volume of 40 µL. Cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension of 72°C for 5 min. The assay was performed on a Bio-Rad-T100 Thermocycler (Bio-Rad Laboratories Pty Ltd., Gladesville, NSW, Australia).

Mycoplasma bovis Serology. Sera were stored at –80°C before evaluation using a commercially available *M. bovis* ELISA kit (BIO K 302; Bio-X Diagnostics, Rochefort, Belgium). The ELISA uses a recombinant

protein from *M. bovis* expressed by *Escherichia coli* as a plate antigen. The assay was performed according to the manufacturer's instructions. Optical density (OD) coefficient (ODC%) was calculated as the proportion of the average positive control OD and was calculated for each sample according to the formula

$$\frac{(\text{OD sample} - \text{OD negative serum})}{(\text{OD positive serum} - \text{OD negative serum})} \times 100.$$

Standard deviation and coefficient of variation were calculated for each sample pair duplicate. Duplicate samples with a coefficient of variation greater than 0.2 and ODC% greater than 20 were rejected and the assay was repeated. According to the manufacturer's directions, sera with an ODC% equal to or greater than 37% were considered positive. An ODC% less than 37% was considered negative.

Statistical Analyses

Statistical analyses were performed using Stata (versions 15 and 16, StataCorp, College Station, TX). For apparent prevalence at weaning, the 95% confidence interval (CI) was calculated with stratification and accounting for clustering by herd of origin of the calves using Stata's "proportion" command with the "svy" prefix. The 2 calving groups from herd 2 were treated as separate herds. For other samplings, exact binomial confidence intervals were calculated using Stata's "cii" command. Variation between herds in seroprevalences at weaning and calving and in proportions seroconverting from precalving to postcalving were assessed using intraclass correlation coefficients calculated using the "xtlogit" command in Stata. For seroprevalences at weaning, the herd of origin of the calves was fitted as the random effect, with the 2 calving groups from herd 2 treated as separate herds. For other analyses, the herd in which the heifers calved was fitted as the random effect. If prevalences or proportions are the same in every herd, the true intraclass correlation coefficient would be 0.00, and if all calves in some herds were positive or seroconverted and no calves in other herds were positive or seroconverted, the true intraclass correlation coefficient would be 1.00. Seroprevalences were compared between

successive samplings using exact McNemar *P*-values, calculated with Stata's "mcc" command. Only calves with ELISA results at both samplings being compared were included. Proportions of heifers seronegative at weaning that had seroconverted by prebreeding (i.e., were seropositive at the prebreeding sampling) were compared between those whose herd of birth had *M. bovis* clinical disease in the milking group and those from the uninfected herd with logistic regression with herd fitted as a random effect using Stata's "xtlogit" command. The estimated sensitivity of serology for detecting *M. bovis* culture positivity at weaning was calculated using the "proportion" command with the "svy" prefix in Stata, with stratification by herd using only those herds with *M. bovis* culture positive calves. One herd had only 1 such calf, so the pooled variance was scaled to account for this. This was the same as applying the average of the variances from the herds with multiple calves to the herd with 1 calf.

Probabilities of *M. bovis* Shedding in Heifer Groups

We estimated probabilities that groups of heifers, reared to weaning in herds with *M. bovis* clinical disease in the milking group (and so potentially exposed to *M. bovis* preweaning), would contain at least 1 shedding heifer postcalving using a Bayesian latent class model, fitted using OpenBUGs (version 3.2.3; an open source equivalent of WinBUGs; Lunn et al., 2000). Under this Bayesian approach, prior probability distributions (which describe the probabilities of various values for the unknown values before the study data are used based on prior knowledge and evidence) are statistically combined with observed data to generate posterior probability distributions (revised probabilities of various values for the unknown values after incorporating the study data).

The model was

$$AP = Se \times TP,$$

where AP is apparent prevalence (i.e., the proportion of heifers postcalving that were culture positive for *M. bovis* on at least 1 postcalving conjunctival, nasal, vaginal, or milk sample); Se is the animal-level diagnostic

Table 2. Primers used for the *Mycoplasma* spp. and *Acholeplasma* spp. conventional PCR

Name	Sequence	Target
MycoF	5'-GGGGATGGATTACCTCCTTT-3'	16S-23S rRNA intergenic spacer region of <i>Mycoplasma</i> spp. (GenBank accession no. AY729934; adapted from Tang et al., 2000)
MycoR	5'-TTCCAGACCCAGGCATC-3'	
AlaiF	5'-AACAAAGGGCACACAGTGGA-3'	16S-23S rRNA intergenic spacer region gene of <i>Acholeplasma</i> spp. (GenBank accession no. AY740437)
AlaiR	5'-CAGGGCTCTCACCTTCTTCG-3'	

sensitivity of the combination of conjunctival, nasal, vaginal, and milk samples for detection of shedding from any site (where the heifer is classified as being culture positive if any of its 4 samples are *M. bovis* positive); and TP is true prevalence (i.e., the actual but unobserved proportion of heifers shedding *M. bovis* postcalving from any site).

The animal-level diagnostic specificity of the combination of samples was assumed to be 1.0 (i.e., 100%). The probabilities that groups of 20, 50, and 100 heifers would contain at least 1 shedding heifer postcalving were calculated as $1 - [(1 - TP)^n]$, where n is the number of heifers in the group.

The data used for the model were the observed apparent prevalence at the individual animal level of *M. bovis* postcalving (from any of the 4 sites) for calves from the 7 herds with clinical *M. bovis* disease (1/315). A relatively uninformative prior distribution was used for sensitivity with only very low values (i.e., values close to 0.0) and very high values (i.e., values close to 1.0) considered unlikely; a mode of 0.5 and 95% probability of <0.94 were used. The prior distribution for true animal-level prevalence was based on one author's experiences with *M. bovis*-exposed replacement heifers in other herds. In these herds, there is typically no evidence that *M. bovis* is present in groups of such heifers, but the true animal-level prevalence is probably not zero, as *M. bovis* clinical disease has occurred in heifers from some herds postcalving with a much higher incidence than in the older cows. This suggests that these heifers had entered the milking herd already subclinically infected rather than acquiring infection from older cows after calving. The prior distribution for true prevalence had a mode of 0.006 (i.e., 0.6%) and 95% probability of <0.10 (i.e., <10%). Convergence was assessed after 10,000 iterations using methods described by Toft et al. (2007). Posterior distributions were based on a further 100,000 iterations. The 95% probability intervals were defined as the 2.5th and 97.5th percentiles of the posterior probability distributions.

RESULTS

Mycoplasma Species Isolation

Mollicutes isolated from the nose, conjunctiva, vagina, colostrum, or milk of all study heifers available for sampling at weaning, prebreeding, precalving, calving, and postcalving are reported in Table 3. Apparent prevalences of *M. bovis* colonization were as follows: weaning, 3.6% (16/450; 95% CI: 2.2–5.7%); prebreeding, 0.5% (2/414; 95% CI: 0.1–1.7%); precalving, 0% (0/374; 1-sided 97.5% CI: 0.0–1.0%); and postcalving, 0.3% (1/356; 95% CI: 0.0–1.6%). *Mycoplasma bovis* was

not isolated from the colostrum of any of the 288 heifers sampled at calving. In calves from the 7 herds with clinical *M. bovis* disease, the apparent prevalence at weaning was 4.0% (16/400; 95% CI: 2.5–6.4%) and the apparent prevalence postcalving was 0.3% (1/315; 95% CI: 0.0–1.8%).

The 19 *M. bovis* isolates were from 17 different heifers, with *M. bovis* detected on only 1 occasion from each of 16 heifers. For 15 heifers, this was at weaning sampling from the nose ($n = 10$), conjunctiva ($n = 3$), and vagina ($n = 2$). For 1 heifer, this was from the nose at prebreeding sampling; this heifer originated in herd 8 (the uninfected herd). *Mycoplasma bovis* was not isolated from this heifer at any other sampling. For the remaining heifer, *M. bovis* was detected from the nose on 3 occasions (at weaning, prebreeding, and postcalving samplings); this heifer originated from herd 1. Study investigators did not detect any clinical signs consistent with mycoplasmosis at the time of heifer samplings, and herd managers did not report any clinical signs in the study heifers consistent with mycoplasmosis to veterinarians servicing the herds, who meet with management on a monthly basis at minimum.

The 16 heifers with *M. bovis* isolated at weaning were from 5 herds. These included herd 1 ($n = 2$), herd 2 ($n = 2$ autumn born and $n = 2$ spring born), herd 3 ($n = 4$), herd 5 ($n = 1$), and herd 6 ($n = 5$). Apparent prevalences of *M. bovis* carriage at weaning ranged from 0% (herds 4, 7, and 8) to 10% (herd 6). There was evidence that apparent prevalences at weaning were clustered to some degree by herd (intraclass correlation coefficient: 0.12 for all herds and 0.07 for herds 1–7 only; 95% CI: 0.01–0.64 for all herds and 0.00–0.70 for herds 1–7 only).

Mycoplasma bovigenitalium was most commonly isolated from the vagina (30/32). Of these, most were at postcalving (21/30). However, several were at weaning (8/30), well before calves had access to bulls. *Mycoplasma bovoculi* was most commonly isolated from the conjunctiva (38/48), of which most were at weaning (37/38). This was followed by the nose (11/48), of which most were also at weaning (9/11). *Mycoplasma bovirhinis* was isolated only from the nose ($n = 13$), of which most were at weaning (12/13).

Acholeplasma spp. were the most commonly isolated *Mollicutes* ($n = 210$). Most were isolated from the nose (133/210) at weaning (14/133), prebreeding (63/133), and precalving (53/133). This was followed by the conjunctiva (66/210) at weaning (9/66), prebreeding (45/66), and precalving (10/66).

A few heifers had 2 different *Mycoplasma* isolates from different sites at the same sample time point. For 8 heifers with *M. bovoculi* isolated from the conjunctiva at weaning, 6 had *M. bovoculi* and 2 had *M. bovis*

Table 3. Number of calves with *Mollicutes* isolated from the conjunctiva, nose, and vagina at weaning, prebreeding, precalving, and postcalving; colostrum at calving; and milk approximately 30 d postcalving¹

Sampling	No. of calves enrolled	Sample site	<i>Mycoplasma bovis</i>	<i>Mycoplasma bovirhinis</i>	<i>Mycoplasma bovovulv</i>	<i>Mycoplasma bovirhinis</i>	<i>Acholeplasma</i> sp.	Unspecified
Weaning	450	Conjunctiva	3	—	37 (2)	—	9 (2)	20 (8)
		Nose	11	—	9 ²	12	14 ² (2)	4
		Vagina	2	8	—	—	1 (1)	—
		Subtotal	16	8	46 {41}	12	24	24 {23}
Prebreeding	414	Conjunctiva	—	—	—	—	46 (2)	—
		Nose	2 (1)	—	1	—	63 (2)	—
		Vagina	—	—	—	—	6	—
		Subtotal	2	—	1	—	115 {99}	—
Precalving	374 [27]	Conjunctiva	—	—	1	—	10 (1)	1
		Nose	—	—	—	—	53 (5)	—
		Vagina	—	1 (1)	—	—	1	—
		Subtotal	—	1	1	—	64 {57}	1
Calving	288 [15]	Colostrum	—	—	—	—	2	—
Postcalving ³	355 ⁴ [24]	Conjunctiva	—	2	—	—	1	3
	356 [24]	Nose	1	—	1	1 ²	3 ²	3 (1)
	355 ⁴ [24]	Vagina	—	21 (6)	—	—	1	2
	356 [24]	Milk	—	—	—	—	—	—
		Subtotal	1	23	1	1	5	8 {7}
Total			19	32	48 {43}	13	210 {187}	33 {31}

¹Calves were from 8 herds (7 herds with *Mycoplasma bovis* clinical disease and 1 uninfected herd). Numbers in () brackets are the number of heifers that originated from herd 8 (the uninfected herd). For example, of the 37 heifers with *Mycoplasma bovis* detected at weaning in the conjunctiva, 2 were from herd 8. Numbers in { } brackets are the subtotal or total number of heifers colonized with the specified isolate after accounting for calves with the same isolate detected from 2 different sample sites at the same sampling. For example, 46 isolates of *M. bovoculi* were detected at weaning from 41 calves. Numbers in [] brackets are the number of autumn-born heifers that did not reach minimum breeding BW and were subsequently bred at the same time as the spring-born heifers. For example, of the 374 heifers sampled precalving, 27 were autumn-born but bred to calve with the spring-born heifers.

²A single heifer had both species isolated from this sample; no other animal had more than 1 species isolated from the same site at the same sampling time point.

³Samples collected approximately 1 mo postcalving.

⁴Vaginal and conjunctiva swabs from 1 heifer were both mistakenly labeled as “vaginal” during collection; *Mycoplasma bovirhinis* was isolated from 1 sample, so results for both samples were excluded from this table.

Table 4. *Mycoplasma bovis* seroprevalences (%; no. in parentheses) in heifers at weaning from the 7 herds with *M. bovis* clinical disease in the milking group (herds 1–7) and the uninfected herd (herd 8)

Herd	Clinical <i>Mycoplasma bovis</i> disease in milking group	<i>Mycoplasma bovis</i> seroprevalence ¹
1	Yes	66 (33/50)
2 (autumn-born heifers)	Yes	86 (43/50)
2 (spring-born heifers)	Yes	98 (48/50)
3	Yes	72 (36/50)
4	Yes	52 (26/50)
5	Yes	28 (14/50)
6	Yes	86 (43/50)
7	Yes	92 (46/50)
1–7 pooled		72 (289/400)
8	No; herd deemed uninfected	2 (1/50)

¹Seroprevalence was clustered by herd. Intraclass correlation coefficient: 0.30 for herds 1–7 and 0.52 for all herds; 95% CI: 0.12–0.57 for herds 1–7 and 0.27–0.76 for all herds.

isolated from their noses. Postcalving, 1 heifer had *M. bovis* isolated from the nose and *M. bovis genitalium* from the vagina.

Mycoplasma bovis Serological Results

Mycoplasma bovis seroprevalences of heifers at weaning by herd are reported in Table 4. Seroprevalences were high (66–98%) in herds 1, 2, 3, 6, and 7 (all herds

with clinical *M. bovis* cases in the milking group), moderate to low (28% and 52%) in herds 4 and 5 (both herds with clinical *M. bovis* cases in the milking group), and very low (2%) in herd 8 (uninfected herd). The 2% seroprevalence for herd 8 was representative of a single heifer with an ODC% of 41.7%, which was marginally above the manufacturer's recommended cut-off value for a seropositive result (>37%).

Mycoplasma bovis ELISA seroprevalences and proportions seroconverting are shown in Table 5. For heifers from herds with *M. bovis* clinical cases in the milking group (herds 1–7), there was a significant decrease in seroprevalence from 72% at weaning to 27% at prebreeding after commingling with other heifers at the common heifer rearing grazing area. For heifers from herds 1 through 7, *M. bovis* seroprevalence further declined to 12% at precalving and then increased slightly to 18% postcalving. The proportions that seroconverted from weaning to prebreeding were 16% for heifers from herds 1 through 7 and 24% for heifers from herd 8. For later periods, proportions that seroconverted were low (6–13%).

Serological results were available at all 4 sampling time points for 324 of the 450 heifers. Of the 209 of these that were seropositive at weaning, 33% (68/209) were seropositive at prebreeding, 14% (29/209) were seropositive precalving, and 21% (44/209) were seropositive postcalving. For these 209 calves, mean time from weaning to prebreeding was 282 d (range between

Table 5. *Mycoplasma bovis* ELISA seroprevalences and proportions seroconverting (%; no. in parentheses) in heifers from the 7 herds with *M. bovis* clinical disease in the milking group and the 1 uninfected herd as well as the duration of period (mean; range between heifers in parentheses)

Measure and sampling or period	Seroprevalence or proportion seroconverting ¹		Duration of period (d)
	7 herds with <i>M. bovis</i> clinical disease	1 uninfected herd	
Seroprevalence			
Weaning	72 ² (289/400)	2 (1/50)	
Prebreeding	27 ^{2,3} (101/368)	24 (11/46)	
Precalving	12 ^{3,4} (41/331)	12 (5/43)	
Postcalving	18 ⁴ (58/315)	17 (7/41)	
Proportions seroconverting ⁵			
Weaning to prebreeding	16 ⁶ (16/100)	24 ⁶ (11/45)	283 (244–296)
Prebreeding to precalving	6 (15/235)	12 (4/33)	278 (249–394)
Precalving to postcalving ⁷	13 (35/277)	8 (3/37)	91 (66–132)

¹Not all study heifers were available for sampling at each of prebreeding, precalving, and postcalving samplings.

²*P* for comparison of these two proportions is < 0.001.

³*P* for comparison of these two proportions is < 0.001.

⁴*P* for comparison of these two proportions equals 0.007.

⁵Proportions of seronegative heifers at the start of period that were seropositive at the end of the period.

⁶*P* for comparison of these two proportions equals 0.230.

⁷Intraclass correlation coefficient for clustering by herd where these 314 heifers calved (*n* = 21 herds) uninformative (0.00; 95% CI 0.00–1.00), but for the 9 herds that received more than 10 heifers, there was minimal variation between herds in proportions seroconverting from precalving to postcalving (7–19%), indicating that there was minimal clustering by herd of calving.

calves: 244–296), to precalving was 552 d (range: 496–584), and to postcalving was 643 d (range: 581–713).

Of the heifers that were *M. bovis* culture positive, 89.5% (17/19) were seropositive. One of the seronegative results was from a heifer at weaning and the other was postcalving from the heifer with *M. bovis* isolated at weaning, prebreeding, and postcalving. Of the 16 calves that were *M. bovis* culture positive at weaning, 15 were seropositive at that time (estimated sensitivity of serology for detecting *M. bovis* culture positivity at weaning: 93.8%; 95% CI: 52.6–99.5%).

Probabilities of *M. bovis* Shedding in Heifer Groups

Posterior probabilities (i.e., distributions of probabilities based on the prior probability distributions and the observed data) that a group of 20, 50, or 100 heifers, from herds with *M. bovis* clinical disease in the milking group and potentially exposed to *M. bovis* preweaning, would contain at least 1 shedding heifer postcalving were 0.239 (95% probability interval: 0.035–0.786), 0.495 (95% probability interval: 0.084–0.979), and 0.745 (95% probability interval: 0.161–1.000) respectively. The posterior probability distribution for true prevalence was median 0.014 (i.e., 1.4%; 95% probability interval: 0.002–0.074) and for animal-level diagnostic sensitivity was median 0.330 (95% probability interval: 0.033–0.937).

DISCUSSION

Owners or managers of dairy herds that experience a *Mycoplasma bovis* clinical mastitis outbreak often question what to do with calves intended to be retained as herd replacements but fed waste milk that may have resulted in exposure to *M. bovis*. In such a scenario, frequently there are clinical *M. bovis* disease and deaths in the preweaned calves. What risk do the surviving replacement heifers pose as a source for ongoing *M. bovis* transmission within or between herds? In a review including studies of preweaned calves from *M. bovis*-infected herds with high *M. bovis* prevalences, Maunsell and Donovan (2009) concluded that calves from infected herds are often colonized with *M. bovis* within days of birth and that the highest prevalences of nasal shedding are in the first 2 mo of life. Little is known about the duration of *M. bovis* nasal shedding from these calves as they mature, and long-term studies are needed to determine the effect of *M. bovis* colonization in young calves on the risk of *M. bovis* infection and disease as adults (Maunsell and Donovan, 2009). Our results show that for each replacement heifer from a herd with *M. bovis* clinical disease in the milking group, there is a very low, but not zero, risk of *M. bovis* coloni-

zation after their first calving. For the heifers that did not get sampled at study end ($n = 94$), it is unlikely that they were culled or died due to mycoplasmosis as no study heifers with clinical signs consistent with mycoplasmosis were reported to servicing veterinarians. Our results also show that, for groups of 50 or more such heifers, there is at least a nontrivial probability that the group will contain at least 1 shedding heifer postcalving.

In herd 8, no animals had ever demonstrated any clinical signs consistent with mycoplasmosis. The high CT value of 38.8 (classified as “caution” by the testing laboratory) for one of the bulk tank milk PCR results could be due to amplicon artifacts (spurious products or primer dimers) or cross-contamination (Caraguel et al., 2011). In light of this, no detection of *M. bovis* from mastitis cases, and an absence of historical *M. bovis* clinical disease, the milking group in this herd was considered uninfected.

Despite widespread *M. bovis* exposure in the heifer calves from the 7 herds with *M. bovis* clinical disease before weaning, as demonstrated by the *M. bovis* ELISA seroprevalence [72% (289/400); range between herds: 28–98%; Table 4], apparent prevalence of *M. bovis* colonization of mucosal surfaces was low [4% (16/400); range between herds: 0–10%] in these heifers at weaning. The majority of *M. bovis* isolations (84%; 16/19) were at weaning. At subsequent samplings, apparent prevalences of *M. bovis* isolation were very low or zero. Similarly, in a herd from the United States, apparent prevalences of *M. bovis* colonization of mucosal surfaces were high in both cows (24%) and replacement animals (calves, young heifers, and mature heifers; 47%) during the first sampling period (5 mo) starting 2 mo after the initial *M. bovis* clinical mastitis case in the milking group (with subsequent cases identified during the first sampling period) and much lower at subsequent quarterly samplings (Punyapornwithaya et al., 2010). The decrease in *M. bovis* apparent nasal prevalence between weaning and prebreeding in the current study also concurs with Bennett and Jasper (1977), where apparent nasal prevalences of *M. bovis* were very high (30–48%) in young animals from *M. bovis*-infected herds up to approximately 1 yr of age and much lower (7–9%) in those aged 12 to 24 mo. Along the same lines, in heifers from the uninfected herd, only 1 heifer was found to have become colonized with *M. bovis* in the nose at prebreeding (and no evidence of *M. bovis* clinical disease) after commingling with heifers from the infected herds. This was despite considerable exposure of these heifers to *M. bovis* between weaning and prebreeding as demonstrated by seroconversion of 24% between weaning and prebreeding. The very low rate of *M. bovis* colonization detection in these heifers at pre-

breeding could have been a result of the length of time (10 mo) between weaning and prebreeding, possibly allowing enough time for natural clearance of any infection. No heifers from the uninfected herd were infected with *M. bovis* at precalving or postcalving despite *M. bovis* seroconversion of 12% between prebreeding and precalving and 8% between precalving and postcalving. Management of heifers in a pasture-based system may have also played a role in minimizing infective doses of pathogen numbers during transmission events, enabling seroconversion but not resulting in any persistent subclinical infections beyond prebreeding. These findings for weaned, extensively managed heifers contrast infection rates in unweaned, close-contact calves in diseased herds, which can be as high as 100% (Maunsell and Donovan, 2009). These differences are likely due to age and more frequent and higher doses of infective pathogen numbers during respiratory aerosol transmission events for the younger unweaned calves.

Diagnostic sensitivity of nasal swabs in our study may have been reduced by the length (15 cm) of the swabs used. In a review of *M. bovis* colonization and shedding in young calves, Maunsell and Donovan (2009) discussed several reports of high nasal prevalences of up to 100% in calves from diseased herds. However, in their own studies of calves fed milk replacer containing *M. bovis*, the palatine and pharyngeal tonsils were the main sites of *M. bovis* infection by 2 wk postinoculation, without detection of significant nasal shedding (Maunsell et al., 2012). This highlights wide variation in potential *M. bovis* nasal prevalences in young calves from diseased herds, possibly linked to infection route (oral ingestion vs. aerosol inhalation). As a result, our nasal apparent prevalences may underestimate the true prevalences of *M. bovis* colonization in the pharynx and upper respiratory tract, especially in the calves at weaning. Further, it is unknown whether and how the distribution of *M. bovis* colonization of upper respiratory anatomical sites [e.g., distal nose, proximal (or deep) nose, tonsil, and nasopharynx] might vary based on infection route and time. For example, as the calf grows, sensitivity may decline with age if *M. bovis* colonization is less common (or organism numbers are less) in the distal nasal passage relative to the proximal nasal passage. In our study, sampling the proximal nasal passage or tonsils in live animals was considered impractical, especially as the calves matured. Apparent prevalences of other identified *Mycoplasma* sp. in the conjunctiva and vagina also declined as the calves got older, except for *M. bovis genitalium*, where the apparent prevalence increased in the vagina postcalving.

One of the *M. bovis* isolations at prebreeding was from a heifer sourced from the uninfected herd. The seroprevalence at weaning for heifers from this herd was

only 2% (compared with 28–98% for the other 7 herds), which represents a single seropositive heifer, providing further evidence that heifers in this herd were not infected before weaning. This single seropositive heifer in this herd at weaning (a different heifer than the animal with *M. bovis* isolated at prebreeding) was marginally seropositive (ODC% of 41.7%) according to the manufacturer's cut-off value (ODC% of 37%). When interpreted in light of no history of *M. bovis* clinical disease and no compelling evidence of infection in this herd from results of other diagnostic tests, this seropositive ELISA result is likely a false positive (i.e., due to imperfect specificity). The seroconversion proportion from weaning to prebreeding for heifers from the uninfected herd was 24%, demonstrating exposure of some of these heifers to *M. bovis* after weaning. Therefore, it is likely that the heifer sourced from this uninfected herd and identified as colonized with *M. bovis* at prebreeding also became infected after weaning due to commingling with infected heifers from other herds. It is unlikely that this was a false-positive culture result because isolates were classified as *M. bovis* based on sequencing results.

Our results highlight the challenges in designing effective biosecurity protocols to prevent introducing *M. bovis* when purchasing replacement heifers. The nose was the most common anatomical location for *M. bovis* isolation (74%; 14/19 isolates) followed by the conjunctiva (16%; 3/19) and the vagina (10%; 2/19). Isolation of *M. bovis* from the nose is also common in young calves from endemically infected herds (Stipkovits et al., 2000; Maunsell and Donovan, 2009). However, in our study, *M. bovis* apparent nasal prevalence was highest at weaning and very low thereafter. Of the 324 heifers that were swabbed at all 4 main samplings, we detected *M. bovis* on multiple occasions from only a single heifer, with detection in its nose at weaning, prebreeding, and postcalving. This finding is consistent with previous work. Punyapornwithaya et al. (2010) did not isolate *M. bovis* more than once from the same individual replacement animal [calves, young heifers, and mature heifers; range total (no.): 76–95] from repeat samplings at quarterly intervals over a 1-yr study period. *Mycoplasma bovis* was not detected from this same study heifer precalving. A colostrum sample is recommended as an *M. bovis* screening sample for newly purchased pregnant replacement heifers (Wilson and González, 1997; Wilson, 1999; Gonzalez and Wilson, 2002). However, *M. bovis* was not isolated from this or any other heifer at precalving samplings or from colostrum upon calving.

The serological results suggest that if the ELISA was used to detect replacement heifer groups that have been exposed to *M. bovis* as calves, it would be most sensitive at a group level if heifers are tested at

weaning (seroprevalence: 72% average; range by herd: 28–98% in herds 1–7). Interestingly, herd 7 had the second highest *M. bovis* seroprevalence (92%) in heifers at weaning despite only 1 *M. bovis* clinical disease case, a cow with arthritis, and no apparent clinical *M. bovis* mastitis cases. This widespread exposure in calves may have been because of widespread subclinical mastitis infections in the milking cows or undetected clinical *M. bovis* mastitis cases. In addition to calves being exposed to *M. bovis*-contaminated colostrum or milk, *M. bovis* may have been transmitted between calves; moreover, mechanical transmission via herd workers or fomites is possible. After weaning, *M. bovis* ELISA group seroprevalences were less useful as an indicator of *M. bovis* exposure as calves, with seroprevalences at prebreeding (27%), precalving (12%), and postcalving (18%) being similar to seroprevalences (6–22%) found in groups of 50 lactating cows in herds with no clinical history of *M. bovis* (Parker et al., 2017a). This low *M. bovis* ELISA seroprevalence at precalving in this study suggests that ELISA is not useful for prepurchase assessment of replacement heifer groups precalving for evidence of previous exposure to *M. bovis* as calves. Only 33% of seropositive heifers at weaning were seropositive at the next sampling (prebreeding), and it is possible that some of these heifers were re-exposed to *M. bovis* postweaning. Therefore, these results suggest that, in the absence of re-exposure, antibodies in the majority of seropositive heifers at weaning do not persist beyond 9 mo. This therefore limits the ability of the BIO K 302 ELISA to detect *M. bovis* exposure in mature heifer replacements that were exposed to *M. bovis* as calves. However, other *M. bovis* ELISA now commercially available may give different results, with a recent study demonstrating greater sensitivity and specificity of an alternative ELISA (ID Screen ELISA; IDVet, Grabels, France) compared with the BIO K 302 ELISA (Andersson et al., 2019). At the individual animal level, the proportion of *M. bovis*-infected heifers that were seropositive at the same time they were detected with *M. bovis* (90% sensitivity) was consistent with our previous findings of 94% in cows with *M. bovis* clinical mastitis (Hazelton et al., 2018).

Mycoplasma bovis clinical disease was not detected in study heifers from weaning to entry into the milking herd and up to the time of postcalving sampling, approximately 1 mo postcalving. Punyapornwithaya et al. (2010) proposed that the risk of clinical mastitis in asymptomatic *M. bovis* carriers may not be high due to differences in virulence of *M. bovis* strains within the same herd, with asymptomatic carriers colonized with avirulent strains and clinical mastitis cases being infected by a virulent strain. However, in Australia, this appears not to be the case. Genetic characterization

of 82 *M. bovis* isolates collected over 9 yr (2006–2015) identified a single *M. bovis* strain circulating throughout Australia with marked genomic similarity between isolates from clinically affected and non-clinical cattle (Parker et al., 2016). Our findings suggest that the risk of *M. bovis* carriage in mature dairy replacement heifers that have been exposed to *M. bovis* preweaning is very low. However, detection of *M. bovis* colonization in the nose of a single heifer at weaning, prebreeding, and postcalving is of importance if eradication is being attempted at both the herd and country levels.

When modeling the probabilities of *M. bovis* shedding in heifer groups of different sizes, the 95% probability intervals were wide, indicating that considerable uncertainty exists about the actual probability that a group of heifers would contain at least 1 shedding heifer postcalving for each of these group sizes. However, for group sizes of 50 and 100, lower limits of probability intervals were not trivial (0.084 and 0.161, respectively). Therefore, if a herd is looking to expand, purchases of 50 or more replacement heifers from herds with *M. bovis* clinical disease in the milking group at the time the heifers were calves present at least a nontrivial risk of *M. bovis* introduction. This emphasizes the importance of knowing the source herd's *M. bovis* history before purchase. For the probabilities model used, we estimated the true animal-level prevalence to be low but not zero for heifers upon entry to the milking herd, suggesting that when *M. bovis* clinical disease has been seen in heifers it was most likely due to the heifers carrying subclinical infection into the herd rather than acquiring it from the cows. An alternative possibility may be for the replacement heifers to be uninfected with *M. bovis* and immunologically naïve, making them more susceptible to clinical infections than the older herd cows.

Our results suggest that it is difficult to accurately determine the herd of origin *M. bovis* exposure history of replacement heifers using nasal, conjunctival, and vaginal swabs for *Mycoplasma* culture and the BIO K 302 ELISA. The more sensitive and specific ID Screen ELISA may be a more suitable *M. bovis* screening test at a group level; however, future studies would need to assess the persistence of *M. bovis* antibodies after exposure to determine its limitations.

It is important to be aware of other *Mycoplasma* spp. that can colonize the conjunctiva, nose, and vagina when conducting diagnostic tests from swab samples of these anatomical locations. Due to the diversity of mycoplasmas that may colonize mucosal sites, speciating isolates is important for the interpretation of their significance. *Mycoplasma bovirhinis* was isolated infrequently from the nose at weaning (2.7%) and once postcalving. *Mycoplasma bovirhinis* can be associated

with respiratory disease but is often considered a commensal and secondary invader (Hirose et al., 2003). *Mycoplasma bovigenitalium* was isolated from a few heifers in the vagina at weaning (1.7%) and precalving (0.3%) and from the conjunctiva (0.6%) and vagina (5.9%) at postcalving. *Mycoplasma bovigenitalium* has been associated with mastitis (Counter, 1978), but its associations with reproductive diseases and reproductive performance are poorly defined (Nicholas et al., 2008). *Mycoplasma bovoculi* was predominantly isolated from the conjunctiva at weaning (8.2%) but was also isolated from the nose at weaning (2%), prebreeding (0.2%), and postcalving (0.3%) and the conjunctiva at precalving (0.3%). *Mycoplasma bovoculi* has been involved with infectious bovine keratoconjunctivitis (Langford and Leach, 1973) but is also considered a commensal of the upper respiratory tract (Alberti et al., 2006) and was not observed to be associated with ocular disease in the study calves. The most frequently isolated *Mollicutes* belonged to the *Acholeplasma* species (most likely *Acholeplasma laidlawii*), and these are commonly considered nonpathogenic (Jasper, 1979).

CONCLUSIONS

In replacement heifer groups exposed to *M. bovis* as calves via milk from infected lactating cows, *M. bovis* prevalence can be very low by the time the heifers join the milking group. However, *M. bovis* was repeatedly isolated from the nose of a single heifer at weaning and prebreeding and after entry to the milking group (postcalving), demonstrating the potential, albeit uncommon, carrier status in replacement heifers potentially exposed to *M. bovis* as calves. These results show that, for each replacement heifer from groups in herds with clinical *M. bovis* disease in the milking group and exposed to *M. bovis* preweaning, there is a very low, but not zero, risk of *M. bovis* colonization after their first calving. With groups of 50 or more such heifers, there is a nontrivial probability that the group will contain at least 1 shedding heifer postcalving. The nose was the predominant anatomical site of *M. bovis* colonization with most *M. bovis* nasal isolations at weaning. Serological assessment at weaning appears to be useful for identifying groups of heifers in which there has been widespread exposure to *M. bovis* preweaning. However, at subsequent samplings, seroprevalences were similar to those observed in cows in herds with no clinical history of *M. bovis*.

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