Mycoplasma species in vaginas of dairy cows before and after exposure to bulls and their association with conception

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ABSTRACT

Mycoplasma species can colonize the urogenital tract of dairy cattle. However, interrelationships between *Mycoplasma* spp. and reproductive performance in dairy herds are unclear. In this study, we measured apparent prevalences of *Mycoplasma* spp. in the vaginas of dairy cows ($n = 629$) pre- and post-bull exposure in dairy herds with and without *Mycoplasma bovis* clinical disease ($n = 5$ herds), and assessed associations between variables describing reproductive performance and consequent *Mycoplasma* spp. isolation. *Mycoplasma* spp. were infrequently isolated from the vagina pre- (1.9%; 12/629) and post-bull (3.2%; 20/629) exposure. Of the mycoplasmas isolated, *Mycoplasma bovis* was isolated most frequently (87.5%; 28/32), followed by *Mycoplasma californicum* (9.3%; 3/32). *Mycoplasma bovis* was only isolated from one cow. We were unable to provide any evidence of venereal transmission of *M. bovis* in cows in *M. bovis*-infected herds that use natural service bulls. There was an insufficient number of cows with *Mycoplasma* spp. in the vagina pre-bull exposure to assess effects on subsequent reproductive performance. Cows that had not conceived before post-bull exposure sampling had much greater odds (odds ratio 14.8; 95% confidence interval 4.2 to 52.3) of having a *Mycoplasma* sp. isolated from the vagina at this time compared with those that had conceived. Also, within those that had conceived, delayed conception increased the odds of having a *Mycoplasma* spp. isolated from the vagina at the post-bull exposure sampling by a factor of 1.62 for every additional week not pregnant. The likely cause of these findings is that cows that remain not pregnant for longer are more likely to be served by a bull (likely repeatedly) and subsequently become colonized with a *Mycoplasma* sp. (mostly *M. bovis*).

talium) through venereal transmission. In dairy herds that use bulls, there is a greater chance of isolating a *Mycoplasma* sp. (mostly *M. bovis*) after a period of bull breedings from the vaginas of cows that have remained nonpregnant for longer during the bull breeding period.

Key words: *Mycoplasma* species, vagina, reproduction, bull

INTRODUCTION

Reproductive performance is critical to the profitability of dairy herds. In seasonally calving pasture-based dairy herds, bulls are commonly used for natural matings for at least part of the breeding period. While some studies have demonstrated associations between the presence of various *Mycoplasma* species in the cow's reproductive tract and reproductive disorders (Doig, 1981; Chander et al., 2001), no such associations were detected in other studies (Erno and Philipsen, 1969; Hirth et al., 1970; Langford, 1975) and the reproductive consequences of *Mycoplasma* spp. infection in dairy cows are poorly defined (Nicholas et al., 2008). However, *Mycoplasma* spp. have been frequently isolated from the genital tracts of bulls (Doig, 1981; Ungureanu et al., 1986) and *Mycoplasma bovis* has been isolated from the bull reproductive tract and semen (Haapala et al., 2018). *Mycoplasma bovis* colonization of the prepuce and semen, and seroconversion (ELISA) of bulls have also been observed during, or soon after, bull use in *M. bovis*-infected dairy herds (Hazelton et al., 2018a). This demonstrates the potential for bulls to contribute to *M. bovis* transmission between cattle. Despite this, no field studies are available reporting the prevalence of *M. bovis* in the reproductive tract of cows in herds naturally infected with *M. bovis* that use natural service bulls, and interrelationships between *M. bovis* and reproductive performance in such herds are unclear. Also, no studies have assessed vaginal *Mycoplasma* spp. prevalence in non-*M. bovis*-infected herds that use natural

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service bulls. Assessment of this potential for venereal transmission of *M. bovis* and other *Mycoplasma* spp. from natural service bulls to cows in *M. bovis*-infected and non-*M. bovis*-infected herds, and any possible inter-relationships with reproductive performance, requires organism prevalence studies in the reproductive tracts of cows pre- and post-bull exposure.

When assessing possible causal associations, the temporal sequence between the exposure and outcome is of primary importance. If an animal became exposed after the outcome event of interest had occurred, that exposure cannot have caused that outcome event. Thus, observed associations where the temporal sequence between exposure and outcome is known are more readily assessed for likely causality than where the temporal sequence is unclear. In some studies, exposure to *Mycoplasma* species occurred before negative reproductive outcomes could have occurred following insemination or intrauterine inoculation with *Mycoplasma*-contaminated semen (Hartman et al., 1964; Hirth et al., 1966; Saed and Al-Aubaidi, 1983). In other studies, *Mycoplasma* species were detected after negative reproductive outcomes had occurred (Panangala et al., 1978; Chander et al., 2001; Petit et al., 2008; Catania et al., 2014). In these latter studies, the temporal sequence is unclear, and it is possible that colonization with *Mycoplasma* species occurred after negative reproductive outcomes had occurred.

For *Mycoplasma* species and reproductive performance in cows, one key question is whether exposure to *Mycoplasma* species reduces subsequent reproductive performance? Although *Mycoplasma* spp. colonization of cows may adversely affect subsequent reproductive performance in herds that use bulls, it is possible that a causal relationship in the reverse direction also occurs. In such herds, cows whose conception is delayed may receive more bull services, increasing the risk of venereal transmission of *Mycoplasma* spp. Thus, both questions are of interest. One of the strengths of longitudinal studies is the greater scope for ensuring that exposure precedes the outcome event(s) of interest. In the longitudinal study we report below, we defined various exposure and outcome variables for analyses in an attempt to address questions about both (1) effects of exposure to *Mycoplasma* species on subsequent reproductive performance in lactating cows, and (2) effects of reproductive performance (the exposure variable) on the likelihood of *Mycoplasma* spp. colonization after a period when bulls ran with the lactating cows.

More recently, serology has been used to demonstrate exposure of herds or individual animals to *M. bovis* (Petersen et al., 2018).

The present study was conducted using 5 herds (3 with *M. bovis* clinical disease, 2 without) belonging to a

multiherd farming business. These herds used a combination of AI and natural service bulls during the breeding period. The objectives of this longitudinal study were (1) to describe and compare apparent prevalences of *Mycoplasma* spp. in the vaginas of lactating cows pre- and post-bull exposure in herds with and without *M. bovis*-associated clinical disease, (2) to assess the potential association between presence of *Mycoplasma* spp. in the vaginas of lactating cows pre-bull exposure (the independent variable) and subsequent conception during the natural service period (the outcome or dependent variable), and (3) to assess potential associations between variables describing occurrence of, and time to, conception during the natural service period (the independent variables) and presence of *Mycoplasma* spp. in the vaginas of lactating cows post-bull exposure (the outcome or dependent variable).

MATERIALS AND METHODS

Herds

A longitudinal study was conducted using 5 commercial pasture-based dairy herds from a multiherd business; 3 herds that had experienced clinical disease due to *M. bovis* before the start of their bull breeding periods and 2 herds with no clinical history of *M. bovis* were selected. Four herds (herds 1, 2, 4, and 5) calved seasonally with all calvings occurring in spring, and 1 (herd 3) used split calving, with calving periods in both spring and autumn. In herds that calve seasonally, all calvings occurred within a single restricted time period each year and there was a single restricted breeding period each year. In the herd using split calving, all calvings occurred within 2 restricted time periods each year, and there were 2 restricted breeding periods each year.

Herd *Mycoplasma bovis* Clinical Histories

Herd records for individual *M. bovis* clinical cases and bulk tank milk *M. bovis* surveillance were accessed to describe the herd's *M. bovis* history. All clinical *M. bovis* cases of mastitis (e.g., watery appearance to the milk, multiple affected quarters, nonresponsive to treatment, or a combination of these) or arthritis (or both) identified by herd staff were confirmed by PCR or culture on 1 occasion. Once any *M. bovis* clinical case was detected in the 3 herds with *M. bovis* clinical disease, it was removed from the milking herd and culled. Metered bulk tank milk samples were collected from the milk tanker at milk pick-up for PCR at a commercial laboratory (Thermo Scientific Pathoproof Major 4.2, Dairy Technical Services, Melbourne, Australia). On one oc-

casion, a bulk tank milk sample was collected from the milk outflow valve for *Mycoplasma* culture at the local government laboratory (Animal Health Laboratory, Prospect, Tasmania).

Cows

Between 65 and 76 heifers and 65 to 77 cows had been selected from each herd for a previous unpublished study using computer-generated random numbers, and these same cows were used in this study. To increase the sample size toward 150 animals per herd, the required number of additional cows was selected systematically (e.g., for an additional 20 cows, every 25th cow from a herd of 500) at the herd's first sampling time point for this study. A total of 730 cows were sampled at the first sampling. To be enrolled, cows must have calved on or between 39 and 103 d before their herd's breeding period start date (d 1). Therefore, the 53 cows that did not calve during this period were excluded, including 6 cows from herd 1, 9 from herd 2, 30 from herd 3, 2 from herd 4, and 6 from herd 5. Herd 3 was over-represented in these exclusions because it was a split calving herd and several of the additionally selected cows had calved in autumn instead of spring. Forty-eight (7%) cows were further excluded for the following reasons: at least one sample (vaginal swab or blood) was not collected (24 cows) at either sampling; duplicate samples with the same animal identity codes (4 cows); duplicate cows with the same animal identity codes within herds (10 cows); cows not pregnancy checked on the 2nd herd pregnancy check day (8 cows); and estimated conception dates 7 or more days after the end of the herd's breeding period (2 cows). Therefore, a total of 629 cows from 5 herds were eligible for analyses.

Breeding Period, Bulls, Cow Sampling, and Pregnancy Diagnosis

For all 5 herds, the breeding period consisted of 50 d of AI (commencing in all herds in late spring 2013 on the same calendar date; d 1), followed by a natural service bull breeding period of between 15 and 49 d.

For all 5 herds, AI was performed by the same commercial breeding company. Natural service bulls for these herds were sourced from a common group of bulls reared and managed separately from the lactating dairy herds within the multiherd business. Natural service bulls from this group were allocated to each herd at a ratio of approximately 1 bull per 30 cows. During the bull breeding period, bulls grazed continually with the entire lactating dairy cow group, being separated only at milking times, and the only inseminations during that period were by natural service. The same bulls within each herd were used for the entirety of the natural service breeding period. Table 1 details sample collection days in relation to the bull breeding period and timing of pregnancy check days. A vaginal swab and blood sample were collected from each cow at the end of the AI period (i.e., pre-bull exposure) in early summer 2013, on or between d 48 and 50, and 1 to 3 d before the herd's bull breeding period start date (i.e., the date that bulls were moved into and retained in the lactating cow herd). Second vaginal swabs and blood samples (post-bull exposure) were collected from the same cows between d 83 and 85 for all herds, 35 to 36 d after first samplings, 32 to 34 d after bulls were first introduced to the herd's lactating group. Due to the variation in length of the bull breeding period across herds, the second samples were collected between 14 d before through to 20 d after the end of the bull breeding period.

For collection of vaginal swabs, the vulva of each cow was cleaned of feces with paper towel before parting of the vulva with a thumb and forefinger, allowing insertion of a 150-mm rayon tip swab (FL Medical, Torreglia, Italy) into the vagina as deep as possible. After sampling, each of these swabs was placed directly into a tube containing Amies transport medium. Blood was also collected from the tail vein into a 10-mL SSST II Advanced Vacutainer Tube (BD, Franklin Lakes, NJ). Swabs and blood samples were refrigerated before transport to the laboratory, arriving within 2 to 3 d.

Each cow was assessed for pregnancy using rectal ultrasound examination on 2 occasions (Table 1), with all cows first examined on or between 2 d before, to

Table 1. Pre- and post-bull sampling days by herd in relation to the bull breeding period and pregnancy check days¹

Herd	Pre-bull sample day	Bull breeding period start and end days (duration in days)	Post-bull sample day	1st pregnancy check day	2nd pregnancy check day
1	48	51–65 (15)	83	93	149
2	50	51–99 (49)	85	99	147
3	49	51–65 (15)	85	94	150
4	49	51–94 (44)	84	92	143
5	48	51–94 (44)	83	95	144

¹Days shown are day number of the breeding period where the first day of the herd's breeding period is d 1.

29 d after, the end of the bull breeding period, and all cows re-examined on, or between, 48 to 85 d after the end of the bull breeding period. At each examination, all cows with no signs of pregnancy on ultrasound were immediately assessed by manual rectal palpation. For those diagnosed pregnant, stage of pregnancy was estimated in weeks and the closest AI dates to that estimated conception date identified. If the estimated conception date was within 7 d of the cow's most recent AI date, this AI date was assigned the conception date, otherwise the estimated conception date was calculated from the estimated stage of pregnancy. When there were 2 AI dates within the 7 d period, the most recent date was used as the conception date. Where results from the 2 examinations differed, results from the second examination were used. Thus, occurrence of pregnancy and estimated conception dates for those that conceived were only for pregnancies retained at least until the second examination. Conception by the post-bull exposure sampling as a binary (yes/no) variable was determined for each cow from their estimated conception date, with cows whose conception date was after the herd's post-bull exposure sampling date classified as "no" as were cows not detectably pregnant at the second examination.

Sample and Laboratory Analyses

Mycoplasma Species Isolation and Identification. The vaginal swabs were cultured using the same protocol as that used by Hazelton et al. (2018a). Briefly, specific *Mycoplasma* agar prepared by Elizabeth Macarthur Agricultural Institute, NSW Department of Primary Industries, NSW, Australia, was used. Agar plates were incubated at 37°C in candle jars for 5 to 10 d before examination for colonies with gross morphology consistent with *Mycoplasma* spp. 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.

PCR. A *Mycoplasma* spp. conventional PCR as described by Parker et al. (2017), which could also amplify *Acholeplasma* spp., was used to determine the identity of culture positive isolates. Reaction mixtures contained 0.25 mM dNTP, 2.5 mM MgCl₂, 1.5 U of GoTaq, 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 30s, 55°C for 30s, 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). Amplified PCR products underwent Sanger sequenc-

ing at the Australian Genome Research Facility Ltd. (Westmead, NSW, Australia) to verify species identification.

A commercially available PCR was used to assess clinical *M. bovis* suspect cases (Thermo Scientific Pathoproof Major 4.2, Dairy Technical Services, Melbourne, Australia).

Serology. Sera were stored at -80°C before serological evaluation using a commercially available *Mycoplasma bovis* ELISA kit (BIO K 302, Bio-X Diagnostics, Rochefort, Belgium). The ELISA utilizes a recombinant protein from *M. bovis* expressed by *Escherichia coli* as a plate antigen. The assay was performed according to the manufacturer's instructions. Briefly, 10 µL of sera was diluted 1/100 using supplied dilution buffer before being added to individual wells with each sample analyzed in duplicate. Positive and negative control sera were run in triplicate. Two wells remained blank for the correction of background. Following incubation at 21°C for 1 h the plate was washed with the supplied washing solution. Conjugate solution (anti-mammalian IgG peroxidase – protein G horseradish peroxidase-labeled) was diluted 1:50 using the supplied dilution buffer and 100 µL was then added to each well and incubated for 1 h at 21°C. Following plate washing, 100 µL of the supplied chromogen solution (tetramethylbenzidine) was added to each well before incubation for 10 min at 21°C. The reaction was subsequently stopped using 50 µL of the supplied stop solution (1 M phosphoric acid) and the absorbance optical density (OD) of each well was read using a Polarstar Optima (450 nm) microplate reader (Polarstar Optima, BMG Labtech, Ortenberg, Germany). Sample OD was adjusted by subtracting the average OD from the blank wells. Each microplate was validated using the specified OD threshold for the positive and negative control sera. Optical density coefficient (ODC%) was calculated as the proportion of the average positive control OD and was calculated for each sample according to the following formula: (OD sample – OD negative serum)/(OD positive serum – OD negative serum) × 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 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

All statistical analyses were performed using Stata (version 15, StataCorp, College Station, TX). Apparent prevalences of *Mycoplasma* spp. were compared be-

tween pre-and post-bull exposure, pooled across herds using the exact *P*-value from McNemar's chi-squared test without stratification by herd, calculated using the *mcci* command in Stata. *Acholeplasma laidlawii* was only isolated on 2 occasions, so it was excluded from analyses. For detection of vaginal colonization, we presume the specificity of the method we used is close to 100% but no estimates are available of the sensitivity of that method, so we have not estimated true prevalences of vaginal colonization.

True prevalences of prior exposure to *M. bovis* were estimated using our observed seroprevalences combined with previously reported probability distributions from latent class models for the sensitivity and specificity of the BIO K 302 ELISA (Andersson et al., 2019). The latent (i.e., unobserved) variable that this ELISA aims to detect has not been precisely defined but would be exposure at any time since birth only if antibodies persist for many years after exposure. To estimate true prevalences, we used Bayesian latent class models fitted using OpenBUGs (version 3.2.3, an open source equivalent of WinBUGS; Lunn et al., 2000). Under this 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). For prior probability distributions for sensitivity and specificity of the BIO K 302 ELISA, we used the posterior distributions from informative priors as reported by Andersson et al. (2019). We used an uninformative [i.e., $\beta(1, 1)$] distribution as the prior probability distribution for all true prevalences. Our observed data were the number of animals tested and number seropositive. 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. Ninety-five percent probability intervals were defined as the 2.5 and 97.5 percentiles of the posterior probability distributions. This approach allowed us to incorporate and specifically document the uncertainty about our true prevalence estimates due to the sampling variation in our observed seroprevalences and the uncertainty about the true values of the sensitivity and specificity of the BIO K 302 ELISA.

Potential determinants of the presence of any *Mycoplasma* sp. in the vagina of lactating cows at the post-bull exposure sampling were assessed using logistic regression with Stata's logistic command. Exposure variables of interest were the binary variable, conceived by date of post-bull exposure sampling, and breeding

period start to conception interval (calculated as conception date minus breeding period start date plus 1 and fitted as a continuous variable). For cows that had not conceived by post-bull exposure sampling date, the mean interval for those that did conceive by that sampling date was used. This allowed estimation of effects of both conceived by date of sampling and the nested variable, breeding period start date to conception interval, in the same model. The binary variable, *Mycoplasma* spp. in the vagina before bull exposure, was also fitted in one model, to remove any confounding due to this variable, and herd was fitted as a fixed effect in both models. Linearity in the logic for the continuous variable, breeding period start to conception interval, was assessed using LOWESS plots.

RESULTS

Description of Herd *Mycoplasma bovis* Clinical Patterns

Results from routine bulk tank milk surveillance with an *M. bovis* PCR (and occasionally culture) and the number of *M. bovis* cases in each herd with timing of their diagnosis in relation to d 1 (breeding period start date) are described below. Herd refers to the herd's lactating group and herd size was defined as the number of cows in the herd's lactating group at the time of the second pregnancy diagnosis.

Herd 1. *Mycoplasma bovis* clinical disease; herd size: 1,064 cows. *Mycoplasma bovis* was not detected in bulk tank milk via PCR on d -426 and -340. On d -48 the bulk tank milk was *M. bovis* PCR-positive with a cycle threshold of 34.7. Joint fluid from a cow with arthritis was *M. bovis* PCR-negative on d -47. *Mycoplasma bovis* was not detected in bulk tank milk via PCR on d -12, 15, 43, 74, and 104. On d 15, 1 cow with arthritis had *M. bovis* detected via culture of joint fluid. A quarter milk sample from a cow with clinical mastitis was *M. bovis* PCR-negative on d 49. On d 130 the bulk tank milk was *M. bovis* PCR-positive with a cycle threshold value of 35.4. No other individual cows had been identified as infected with *M. bovis* via PCR or culture before, or during, the study.

Herd 2. *Mycoplasma bovis* clinical disease; herd size: 274 cows. The bulk tank milk was *M. bovis* PCR-positive on d -48, -13, and 15. Seven and 14 cows with clinical mastitis were *M. bovis* PCR-positive on d -9 and 6, respectively, in quarter milk samples.

Herd 3. *Mycoplasma bovis* clinical disease; herd size: 1,066 cows. Bulk tank milk was *M. bovis* culture-positive on d -83 and *M. bovis* PCR-positive on d -81 and d 43. Quarter milk samples from 81 clinical mastitis cases and a joint sample from a cow with arthritis

case were *M. bovis* PCR-positive between d -145 and -47. Between d -88 and -53, quarter milk samples from 16 clinical mastitis cases, joint samples from 3 arthritis cases, and composite milk samples from 2 high SCC (>200 000 cells/mL) cases were *M. bovis* culture-positive.

Herd 4. No *Mycoplasma bovis* clinical disease, single bulk tank milk sample *M. bovis* PCR-positive; herd size: 355 cows. No clinical signs of *M. bovis* infection had ever been recognized in this herd. *Mycoplasma bovis* was not detected in bulk tank milk via PCR on d -425, -340, and -12. On d 15 the bulk tank milk was *M. bovis* PCR-positive with a cycle threshold value of 34.1. In response to this result, composite milk samples from each cow (n = 11) with SCC >200,000 cells/mL were collected on d 24 and tested using PCR and culture. *Mycoplasma bovis* was not detected in any sample. A follow-up bulk tank milk PCR on d 43 was negative for *M. bovis*. Composite milk samples from another 9 cows with clinical mastitis on d 49 were negative for *M. bovis* via PCR, as were bulk tank milk samples collected on d 74, 104, and 130. No individual animal in this herd had ever been confirmed to be infected with *M. bovis* via PCR or culture.

Herd 5. No *Mycoplasma bovis* clinical disease and no evidence of subclinical *M. bovis* infection; herd size: 513 cows. No clinical signs of *M. bovis* infection had ever been recognized in this herd. Bulk tank milk *M. bovis* PCR was negative on all 9 occasions when assessed (on d -426, -340, -48, -13, 15, 43, 74, 104, and 130). No cows had ever been identified as being infected with *M. bovis* via PCR or culture.

Mycoplasma Species Isolated

The *Mycoplasma* spp. isolated from the vaginas of cows pre- and post-bull exposure are detailed in Table 2. *Mycoplasma bovis* was only isolated from the vagina of 1 cow at one sampling (in herd 2 pre-bull exposure). Apparent prevalences of *M. bovis* in the vagina pre-bull exposure were 0.2% (1 in 629 cows) in all herds and 0.3% (1 in 344 cows) in herds with *M. bovis* clinical disease. No cow had more than one of the reported species isolated at the same sampling and only one cow had a *Mycoplasma* sp. isolated at both samplings; 1 cow in herd 3 had *M. bovis* isolated pre- and post-bull exposure. Another cow in herd 3 had *A. laidlawii* isolated pre-bull exposure and *M. bovis* isolated post-bull exposure.

The apparent prevalences of any *Mycoplasma* sp. were similar pre- and post-bull exposure and both prevalences were low (pre: 1.9% or 12/629; post: 3.2% or 20/629; $P = 0.201$).

Table 2. Apparent prevalences (no. of cows positive on culture) of *Mycoplasma* species and *Acholeplasma laidlawii* in vaginas of lactating cows, proportions of *Mycoplasma bovis* ELISA-positive (no. of cows testing positive), and estimated true prevalences of prior exposure to *M. bovis* pre- and post-bull exposure (pre-bull and post-bull, respectively)¹

Herd	<i>M. bovis</i> clinical disease history	No. of cows sampled	Sample collection ¹	Vaginal isolates, % (no. of cows positive on culture)					<i>M. bovis</i> ELISA-positive, % (no. of cows testing positive)	True prevalence of prior exposure to <i>M. bovis</i> , median (95% probability interval)
				<i>Mycoplasma bovis</i>	<i>Mycoplasma bovis</i>	<i>Mycoplasma californicum</i>	<i>Acholeplasma laidlawii</i>	<i>M. bovis</i> ELISA-positive, % (no. of cows testing positive)		
1	Clinical disease	122 ²	Pre-bull ²	3.3 (4)	0	0	0	25.4 (31)	39.6 (20.2 to 61.8)	
			Post-bull	4.1 (5)	0	0	0	24.6 (30)	37.4 (18.4 to 59.6)	
2	Clinical disease	124	Pre-bull	0	0.8 (1)	0	0.8 (1)	26.6 (33)	42.7 (23.1 to 65.2)	
			Post-bull	0.8 (1)	0	0.8 (1)	0	15.3 (19)	14.2 (1.6 to 32.8)	
3	Clinical disease	98	Pre-bull	2.1 (2)	0	1.0 (1)	1.0 (1)	23.5 (23)	34.8 (14.2 to 58.9)	
			Post-bull	10.2 (10)	0	1.0 (1)	0	22.5 (22)	32.2 (11.9 to 56.2)	
4	No clinical disease	144	Pre-bull	2.1 (3)	0	0	0	8.3 (12)	3.5 (0.1 to 14.5)	
			Post-bull	0	0	0	0	4.2 (6)	1.8 (0.1 to 8.9)	
5	No clinical disease	141	Pre-bull	0.7 (1)	0	0	0	13.5 (19)	10.1 (0.7 to 26.3)	
			Post-bull	1.4 (2)	0	0	0	14.2 (20)	11.5 (1 to 28.3)	
	Total	629	Pre-bull	1.6 (10)	0.2 (1)	0.2 (1)	0.3 (2)	18.8 (118)	21.8 (12.4 to 31.6)	
			Post-bull	2.9 (18)	0	0.3 (2)	0	15.4 (97)	13.2 (4.2 to 22.3)	

¹No cow had more than one of these species isolated at the same sampling.

²For a further one of the 122 cows, the genus and species for an isolate from the pre-bull exposure sample could not be determined.

Mycoplasma Species in the Vagina and Reproductive Performance

The crude association between presence of *Mycoplasma* species in the vagina (the independent variable) and reproductive performance (the outcome or dependent variable) was examined. Disregarding timing (pre- or post-bull exposure) of *Mycoplasma* spp. detection in the vagina and timing of conception, at the end of the breeding period, pregnancy did not occur in 10/31 (32.3%) cows with at least one *Mycoplasma* spp. positive sample, and 63/598 (10.5%) cows where no *Mycoplasma* spp. were detected at either sampling. *Mycoplasma* spp. were detected from at least one sampling in 10/73 (13.6%) nonpregnant cows, and 21/556 (3.4%) pregnant cows. Causality underlying this crude association was explored accounting for the temporal sequence of conception and timing of *Mycoplasma* spp. detection in the vagina.

Mycoplasma Species in the Vagina of Cows Pre-Bull Exposure and Subsequent Reproductive Performance

The association between presence of *Mycoplasma* species in the vagina of cows pre-bull exposure (the independent variable) and subsequent reproductive performance (the outcome or dependent variable) was examined. In total, 152 cows had not conceived on or before the pre-bull exposure sampling date and so were available for assessing effects of presence of *Mycoplasma* spp. in the vagina on subsequent reproductive performance. Of these, only 2/152 (1.3%; 1 *M. bovis* and 1 *M. bovis*) had a *Mycoplasma* sp. detected in their vagina pre-bull exposure. Accordingly, there was an insufficient number of cows with *Mycoplasma* spp. in the vagina pre-bull exposure to assess effects on subsequent reproductive performance.

Reproductive Performance and Presence of Mycoplasma Species in the Vagina of Cows Post-Bull Exposure

The association between reproductive performance (the independent variable) and presence of *Mycoplasma* species in the vagina of cows post-bull exposure (the outcome or dependent variable) was examined. Results of analyses of potential determinants of presence of *Mycoplasma* spp. in the vagina post-bull exposure are shown in Table 3. Herd 4 had no cows with a vaginal *Mycoplasma* spp. post-bull exposure, so all 144 cows from this herd were excluded from this analysis. Of the cows in the remaining herds, 20/485 (4.1%; 18 *M. bovis* and 2 *M. californicum*) had *Mycoplasma*

spp. detected in the vagina post-bull exposure. Of these 20 cows, only 1 had *Mycoplasma* spp. in the vagina detected pre-bull exposure (a cow where *M. bovis* was detected at both samplings). Odds of isolating a *Mycoplasma* sp. from the vagina post-bull exposure were greater for cows that had not conceived on or before the post-bull exposure sampling date [odds ratio (OR) 14.8; 95% CI 4.2 to 52.3]. In addition, for cows that conceived on or before the post-bull exposure sampling date, for every additional week that conception was delayed, the odds of having a *Mycoplasma* sp. in their vagina at post-bull exposure sampling were estimated as increasing by a factor of 1.62 (95% CI 1.28 to 2.04).

Serology

Table 2 displays the *M. bovis* seroprevalences (total and by herd) pre-bull exposure and post-bull exposure. Seroconversion incidence in cows from pre- to post-bull exposure (i.e., proportions of cows seronegative pre-bull exposure that were seropositive post-bull exposure) was 3.9% (20/511) and seroconversion incidences by herd were 7.7% (herd 1; 7/91), 3.3% (herd 2; 3/91), 2.7% (herd 3; 2/75), 0.8% (herd 4; 1/132), and 5.7% (herd 5; 7/122).

True prevalences of prior exposure to *M. bovis* are also shown in Table 2, and for the herds with a clinical disease history of *M. bovis* (herds 1–3), true prevalences ranged from 14.2% (95% probability interval 1.6 to 32.8; post-bull exposure herd 2) to 39.6% (95% probability interval 20.2 to 61.8; pre-bull exposure herd 1). For herds with no *M. bovis* clinical disease (herds 4 and 5), true prevalences ranged from 1.8% (95% probability interval 0.1 to 8.9; post-bull exposure herd 4) to 11.5% (95% probability interval 1 to 28.3; post-bull exposure herd 5).

DISCUSSION

Mycoplasma bovis causes important clinical disease in dairy cows, including mastitis, arthritis, and pneumonia. However, even though *M. bovis* has been demonstrated to colonize the reproductive tract of cows (Hirth et al., 1966; Stipkovits et al., 2000; Hazelton et al., 2018b), the reproductive consequences of *M. bovis* and other *Mycoplasma* spp. in dairy cows are poorly defined (Nicholas et al., 2008). To our knowledge, no reports have been published that demonstrate substantial effects of *M. bovis* on herd reproductive performance. Infertility with varying degrees of salpingitis, endometritis, and ovarian adhesions was observed in 4 out of 12 experimentally infected heifers (Hirth et al., 1966), and *M. bovis* has been isolated from aborted fetuses

Table 3. Determinants of presence of *Mycoplasma* species in the vagina of lactating cows (excluding herd 4) at post-bull exposure sampling

Variable	No. of cows	% (no.) with <i>Mycoplasma</i> species in vagina	Adjusted odds ratio ¹	95% CI	P-value ²
Conceived on or before post-bull exposure sampling date ³					<0.001*
Yes	418	2.6 (11)	Referent		
No	67	13.4 (9)	14.8	4.2 to 52.3	<0.001
Interval from breeding period start date to conception ^{3,4}					<0.001*
	485	4.1 (20)	1.62 ⁵	1.28 to 2.04	<0.001
<i>Mycoplasma</i> species detected in vagina at pre-bull exposure sampling					0.540*
No	476	4.0 (19)	Referent		
Yes	9	11.1 (1)	2.4	0.2 to 31.4	0.517
Herd					<0.001*
1	122	4.1 (5)	Referent		
2	124	1.6 (2)	0.2	0.0 to 1.2	0.087
3	98	11.2 (11)	3.6	1.1 to 11.7	0.034
5	141	1.4 (2)	0.3	0.1 to 1.8	0.188

¹All 4 variables were simultaneously fitted in the same model so all odds ratio estimates are adjusted for each of the other 3 variables.

²P-values with asterisks are overall likelihood ratio test P-values; P-values without asterisks are Wald P-values for assessing the respective category relative to the reference category.

³For conceptions retained until at least the second pregnancy examination.

⁴Only conceptions by post-bull exposure sampling date were used; see text for method for cows not conceived by then.

⁵The odds of presence of *Mycoplasma* species at the post-bull exposure sampling were estimated as increasing by a factor of 1.62 for each additional week that conception was delayed.

and dams (Stalheim and Proctor, 1976; Hassan and Dokhan, 2004). Prevalences of *M. bovis* in cows' lower reproductive tracts are commonly low. In the current study, the apparent prevalence of *M. bovis* in the vagina pre-bull exposure was only 1/629 cows (or 1/344 cows in herds having *M. bovis* clinical disease), and *M. bovis* was not detected from vaginas of any cow post-bull exposure. Similarly low vaginal *M. bovis* prevalence (1/42 cows from 5 *M. bovis*-infected herds) has also been demonstrated in another study (Feenstra et al., 1991). Trichard and Jacobsz (1985) and Petit et al. (2008) attempted to isolate *M. bovis* from the lower reproductive tracts from small numbers of cows across 151 and 196 herds, respectively, and detected no *M. bovis* and 1/261 cows with *M. bovis*, respectively, for sampled cows pooled across herds. Assuming many of these herds were not infected with *M. bovis*, cow-level prevalences of *M. bovis* in *M. bovis*-infected herds would be expected to be greater than reflected in these results.

Gille et al. (2018) found that the use of bulls was a risk factor for a dairy herd having a *M. bovis* PCR-positive or ELISA-positive bulk tank, likely because bulls can introduce *M. bovis* into herds, with the suggestion that bulls may be capable of transmitting *M. bovis* to cows within herds. Haapala et al. (2018) describe introduction of *M. bovis* into 2 closed herds via processed semen. However, in the current study, while only 1/344 cows was detected with *M. bovis* in the vagina pre-bull exposure, if bulls that become colonized

from cows are then contagious for some time, the effect of a very low true prevalence in cows pre-bull exposure could be large. However, post-bull exposure no cows had *M. bovis* detected in their vaginas and there was minimal seroconversion to *M. bovis* in cows from the 3 herds with a history of *M. bovis* clinical disease, suggesting that this risk may be low. Given these herds are part of a multiherd business, movement of animals (especially bulls) between herds was probably more common than for other herds. Added to this, the detection of a highly contagious infectious pathogen like *M. bovis* in several herds, one might expect bulls sourced from a common pool and used for natural service to be high risk for potential venereal transmission. This potential was confirmed by a previous study of ours where despite frequent seroconversion, a small number of bulls (3.4%; 4/118) had *M. bovis* detected in their semen and prepuce after use for natural matings in 4 *M. bovis*-infected herds that were part of a multiherd business (Hazelton et al., 2018a). However, our results in this current study provide no evidence from the cows for widespread venereal transmission in 3 *M. bovis*-infected herds that use natural service bulls and are also part of the same multiherd business as Hazelton et al. (2018a). This raises the possibility that the cow with *M. bovis* isolated from its vagina in the current study may have become infected in this location by a route other than venereal transmission. In other studies, *M. bovis* was isolated from the vagina in 3 out of 16 cows

with clinical *M. bovis* mastitis (Hazelton et al., 2018b), and the single cow described by Feenstra et al. (1991) with a vaginal *M. bovis* isolate had also previously had *M. bovis* mastitis. Therefore, *M. bovis* colonization of the vagina may more likely be associated with clinical or subclinical infection elsewhere in the body, rather than with venereal transmission from bulls or from artificially inseminated semen.

While *M. bovis* is the *Mycoplasma* spp. of most interest due to its high virulence, other *Mycoplasma* spp. were isolated more frequently from the vagina of cows. Other *Mycoplasma* spp. apparent prevalences in the vagina were low and similar pre- (1.9%) and post-bull (3.2%) exposure. *Mycoplasma bovis* had the highest apparent prevalences at pre- (1.6%) and post-bull (2.9%) exposure, and *M. californicum* was isolated from 3 cows [1 (0.2%) pre-bull, 2 (0.3%) post-bull exposure]. Only 1 cow had a *Mycoplasma* sp. isolated at both samplings (*M. bovis* at both samplings). Discordance between pre- and post-bull exposure *Mycoplasma* spp. isolations may have been because these organisms do not persist in the vagina, or because organism numbers vary and sometimes are below the detectable limit (Parker et al., 2017).

There was an insufficient number of cows that had not conceived on or before pre-bull exposure with *Mycoplasma* spp. in their vaginas at pre-bull exposure to assess effects on subsequent reproductive performance.

While *Mycoplasma* spp. colonization of cows may adversely affect subsequent reproductive performance, in herds that use natural service bulls we assessed whether there is a causal relationship in the reverse direction. In such herds, cows whose conception is delayed may consequently receive more bull services, increasing the risk of venereal transmission of *Mycoplasma* species and, subsequently, vaginal colonization. Our results are consistent with bulls transmitting *M. bovis* venereally. The odds of having a *Mycoplasma* sp. in the vagina at the post-bull exposure sample date were much greater for cows that had not conceived on or before the post-bull exposure sample date (OR 14.8). For those cows with a delayed conception, odds of having a *Mycoplasma* sp. in their vagina increased by a factor of 1.62 for every additional week not pregnant. These associations largely reflected patterns for *M. bovis*, the *Mycoplasma* sp. in 18 of the 20 colonized cows post-bull exposure that were included in these analyses. *Mycoplasma bovis* is commonly present in bulls' reproductive tracts (Doig, 1981; Hazelton et al., 2018a). One limitation of this result is that our study design did not allow us to identify the precise timing of the colonizations detected at the post-bull exposure sampling. However, most of these colonizations were likely acquired after the pre-bull exposure sampling;

of the 20 cows colonized at the post-bull exposure sampling, only one had *Mycoplasma* spp. in the vagina detected pre-bull exposure (*Mycoplasma bovis* at both samplings). Further, the estimated OR for being colonized at the post-bull exposure sampling were similar when the 9 cows that were colonized at the pre-bull exposure sampling were excluded. This provides evidence that the relationship we observed was due to effects of delayed conception on subsequent risk of vaginal colonization rather than effects of vaginal colonization of subsequent reproductive performance. We adjusted for herd so our observed associations were not confounded due to herd-level variables, or to cow-level variables closely associated with herd. It is likely that the diagnostic sensitivity of vaginal swabs is imperfect, and this could have biased our OR estimates. However, the resulting measurement errors in classification of cows as being colonized with *Mycoplasma* spp. at the post-bull exposure sampling would have been nondifferential (i.e., the error patterns would have not been related to our 2 exposure variables, conceived on or before post-bull exposure sampling date and interval from breeding period start date to conception). Accordingly, due to this bias, our OR estimates may be underestimates of the true effects.

Another limitation of the study is the timing of the post-bull exposure sampling. The plan was for this sampling to be done between 2 1/2 to 3 wk after the end of the natural service bull breeding period (and this was done for herds 1 and 3). This design was chosen to allow time for seroconversion from any possible *M. bovis* exposure up to the last day of the bull breeding period. However, this time between the end of the natural service bull breeding period and post-bull sample collection may have influenced the vaginal *Mycoplasma* sp. apparent prevalences observed for herds 1 and 3. This time may have allowed time for natural clearance of the *Mycoplasma* species from the vagina or reduction of organism numbers to below detectable limits of culture. Therefore, our post-bull exposure *Mycoplasma* sp. apparent prevalences for these 2 herds may be lower than had we sampled during or on the last day of the bull breeding period. In herds 2, 4, and 5, the natural service bull breeding period was unexpectedly extended and therefore the bulls remained within these herds at the time of the (planned) post-bull exposure sampling.

Apparent prevalences of vaginal colonization with *Mycoplasma* spp. post-bull exposure also differed by herd, with much higher prevalence in herd 3. This increased odds of colonization was evident after adjusting for whether or not each cow had conceived on or before post-bull exposure sampling date, and for those that had conceived, their interval from breeding period start date to conception. The reason for this is not easily

explained, but it may have been because while non-pregnant, cows in this herd were served by bulls more often compared with cows in other study herds, or the bulls used in this herd had a higher prevalence of *M. bovis genitalium* colonization in their reproductive tracts than the bulls used in the other herds.

CONCLUSIONS

Prevalences of *Mycoplasma* spp. in the vaginas of cows were low pre- and post-bull exposure, and *M. bovis* was uncommon in cow vaginas in herds with *M. bovis* clinical disease. We were unable to provide any evidence of venereal transmission of *M. bovis* in cows in *M. bovis*-infected herds that use natural service bulls. In cows, vaginal colonization with *M. bovis* may be more likely to be transmitted by routes other than venereal transmission. There was an insufficient number of cows with *Mycoplasma* spp. in the vagina pre-bull exposure to assess effects on subsequent reproductive performance. In dairy herds that use bulls, there is a greater chance of isolating a *Mycoplasma* sp. (mostly *M. bovis genitalium*) after a period of bull breedings from the vaginas of cows that have remained nonpregnant for longer during the bull breeding period. A likely explanation for these results is that cows that remain not pregnant for longer are more likely to be served by a bull (likely repeatedly), and therefore more likely to acquire *M. bovis genitalium* through venereal transmission.

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