J. Dairy Sci. 101:1–6
https://doi.org/10.3168/jds.2017-13512

Short communication: Shedding of *Mycoplasma bovis* and antibody responses in cows recently diagnosed with clinical infection

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ABSTRACT

Mycoplasma bovis can have significant consequences when introduced into immunologically naïve dairy herds. Subclinically infected carrier animals are the most common way that *M. bovis* is introduced into herds. Although *M. bovis* udder infections can be detected by milk sampling lactating animals before their introduction, currently, no definitive way of identifying *M. bovis* carrier animals that are nonlactating (i.e., calves, heifers, dry cows, or bulls) is available. Understanding the prevalence of *M. bovis* shedding from various body sites in clinically infected animals could inform strategies for the detection of subclinical infection in nonlactating stock. The mucosal surfaces of the nose, eye, and vagina of 16 cows with recent clinical mastitis caused by *M. bovis* were examined for the presence of *M. bovis* shedding. Blood was collected for serological evaluation by a commercially available ELISA. *Mycoplasma bovis* was isolated from the vagina of only 3 (18.8%) of the cows and was not detected from the noses or eyes of any of the cows. Fifteen of the 16 (93.8%) cows were seropositive to the ELISA. With such low prevalence of detection of *M. bovis* from the vagina and no detections from the noses or eyes of recently clinically infected animals, it is very likely that sampling these sites would be ineffective for detecting subclinical infection in cattle. Serology using the ELISA may have some use when screening animals for biosecurity risk assessment. However, more information regarding time to seroconversion, antibody longevity, and test diagnostic sensitivity and specificity are required to define the appropriate use of this ELISA for biosecurity purposes.

Key words: *Mycoplasma*, mastitis, shedding, dairy cow

Short Communication

Mycoplasma bovis is one of the major pathogens of biosecurity significance to a dairy herd due to its highly contagious nature and unresponsiveness to most treatments (Fox, 2012). Generally, *Mycoplasma* species can cause disease at several different anatomical locations including the mammary gland, joints, respiratory (Pfützner and Sachse, 1996) and urogenital tracts (Ruhnke, 1994), eye (Alberti et al., 2006), and inner ear (Maunsell et al., 2012). Clinical diagnosis is typically based on clinical presentation with confirmation achieved via culture or PCR of milk, joint fluid, swabs, or tissues. Several molecular diagnostic assays (PCR, loop-mediated isothermal amplification) aimed at detecting various *Mycoplasma* spp. have been described in the veterinary literature. However, in Australia, most commercially available PCR assays are specific for detection of *M. bovis*.

In infected dairy herds, not all infected animals display clinical signs of disease. Understanding the prevalence of *M. bovis* shedding from various body sites is a logical first step in the development of any sampling and testing protocol designed for detection of subclinically infected animals. Little conclusive research is available on the prevalence of *M. bovis* shedding at different anatomical locations in clinically infected animals. Detecting carrier (i.e., subclinically infected) animals is difficult because there is no consistently infected body site to sample. Gonzalez et al. (1992) and Biddle et al. (2003) have highlighted the challenge in detecting subclinical mastitis due to intermittent shedding of *M. bovis* in milk. Furthermore, detecting subclinical infection in nonlactating stock poses an additional challenge.

Initial studies showed experimental inoculation of the pathogenic mycoplasma strain 01 (later identified as *M. bovis*) in the udder resulted in frequent but intermittent shedding of the same strain from the nose, eye, rectum, vagina, and urine of the inoculated cows (Jain et al., 1969). The same mycoplasma strain was also iso-

Received July 17, 2017.
Accepted August 24, 2017.
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lated from the lymph nodes, gastrointestinal contents, uterus, lung, liver, kidney, spleen, joint fluid, and fetus of the inoculated cows at postmortem. In a study by Jasper et al. (1974), following the recent diagnosis of mycoplasma mastitis in 6 cows, *M. bovimastitidis* (later reclassified as *M. bovis*) was isolated from the nose of 1 and vagina of 2 of these cows. Following the culling of these 6 cows, the remaining 112 cows in the herd were all culture negative for *M. bovis* in milk samples, but 1 cow returned an *M. bovis* positive nose swab, and 5 cows returned *M. bovis* positive vaginal swabs. A study by Biddle et al. (2005) demonstrated colonization of body sites other than the mammary gland, including the urogenital and respiratory tracts in 7 cows with *Mycoplasma* spp. udder infection that were sampled once weekly for 4 wk. However, prevalences of detection at each of the samplings were not reported and the nature of the mycoplasma infections (subclinical or clinical) was not described. Punyapornwithaya et al. (2010) investigated a herd experiencing clinical mastitis cases and isolated the same *M. bovis* strain from the mucosal surfaces of subclinical carriers in at least one nose, eye, ear, or vulvovaginal tract swab from 21% of the cows and 47% of the replacement stock sampled once over a 5-mo period commencing soon after the initial identification of clinical mastitis cases in the lactating cows. Three clinical cases (mastitis in 2 cows and polyarthritis in a calf) of *M. bovis* infections were diagnosed during the 5-mo period. Three subsequent samplings of each animal remaining within the herd over the following 10-mo period demonstrated a dramatic drop in *M. bovis* apparent prevalence to below 6.4% in both cohorts at each sampling occasion.

Dairy herds often expand or introduce new genetics by purchasing nonlactating stock (calves, heifers, dry cows, or bulls). As *M. bovis* can be detected from body sites other than the mammary gland, such purchases pose a risk for the introduction of *M. bovis*. More recently, studies have evaluated the use of ELISA to detect animals previously exposed to *M. bovis* (and hence, potentially infected; Wawegama et al., 2016).

Enhanced understanding of the anatomical locations of *M. bovis* infection and serological responses of clinically infected animals could help guide further development of sampling protocols aimed at increasing the sensitivity of detection of *M. bovis* in subclinically infected nonlactating stock. From this, an accurate assessment of biosecurity risk due to purchased stock could then be determined. Therefore, the objectives of this study were (1) to evaluate the frequency of *M. bovis* detection from readily accessible mucosal sites, and (2) to measure the serologic response to *M. bovis*, both in adult dairy cattle with recent clinical *M. bovis* mastitis.

During an *M. bovis* mastitis outbreak in a split calving (spring and autumn) dairy herd, 16 milking cows previously diagnosed with *M. bovis* mastitis via PCR (Thermo Scientific Pathoproof Major 4.2, Dairy Technical Services, Melbourne, Australia) were awaiting expiration of drug withhold periods before being sent for slaughter. These cows had been dried off and separated from the milking herd in the days before sampling for this study. The original milk samples used for *M. bovis* diagnosis had been collected 13 d before sampling for this study for 13 of the cows and 7 d prior for the other 3 cows. Swabs (15 cm rayon tip contained in Amies transport medium; FL Medical, Torreglia, Italy) were collected from the right nostril (nose), one conjunctival sac (eye), and the vagina of each cow, and blood was collected from the tail vein. The type of swab was selected for its affordability and accessibility and the sampling technique was designed for practical application in the field. The samples were chilled in a refrigerator and transported on ice until they reached the laboratory within 2 to 3 d. The collection of all samples had the approval of the University of Sydney Animal Ethics Committee (protocol number 2013/6046).

The swab samples were cultured on selective medium made with Mycoplasma agar [Mycoplasma agar base (Oxoid CM0401, Waltham, MA); Milli-Q water; 0.2% wt/vol calf thymus DNA (Sigma D1501, Sigma, St. Louis, MO); Mycoplasma Selective Supplement G (Oxoid SR0059C); prepared by the Elizabeth Macarthur Agricultural Institute; New South Wales Department of Primary Industries, NSW, Australia]. Agar plates were then incubated at 37°C in candle jars with elevated CO_2 levels for 5 to 10 d. Following incubation, the plates were examined for colonies with gross morphology consistent with *Mycoplasma* species. For each isolate, several colonies were selected and aseptically transferred into 40 μL of sterile PBS. The PBS suspension was vortexed and 10 μL subcultured onto mycoplasma agar. Colonies from the subculture plates were subsequently harvested and stored in Cryobead tubes (Protect Multipurpose; Thermo Fisher Scientific, Waltham, MA) at −80°C. The residual 30 μL of PBS was stored at −20°C pending PCR evaluation to identify the species of *Mycoplasma* present.

Up to 3 PCR assays were used to determine the identity of the culture isolates. First, isolates were processed using a *Mycoplasma* spp. conventional PCR assay as described by Parker et al. (2017). This was followed by an *Acholeplasma laidlawii* conventional PCR assay designed using the Primer3 software program (Untergasser et al., 2012; Table 1). For each PCR assay, reaction mixtures contained 0.25 m*M* deoxynucleotide triphosphates, 2.5 m*M* $MgCl_2$, 1.5 U of GoTaq, 0.25 μ*M*