Mycoplasma as an Environmental Isolate on Dairy Farms

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Abstract

Mycoplasma bovis is an important bovine pathogen causing mastitis, pneumonia, arthritis, and metritis. Mycoplasma infections are considered contagious, with transmission from cow to cow as a contagious mastitis spread at milking time or as a respiratory disease. As part of follow up to an earlier mycoplasma prevalence study, this study evaluated the possibility of mycoplasmas as environmental pathogens on dairy farms with mycoplasma mastitis affecting their herds. During a clinical mastitis outbreak caused by *Mycoplasma* spp, a sample of recycled sand bedding from lactating cow housing areas was culture-positive for mycoplasma. Additional sampling found 14/20 sand-bedded pens positive for *Mycoplasma* spp on the same farm. *Mycoplasma* spp were subsequently isolated from other used bedding samples from two other farms with mycoplasma mastitis in their herds; all positive beddings were sand, with the exception of straw bedding from one pen. Presence of *M. bovis* was verified with PCR testing of samples from two of the three farms; no PCR testing was done on samples from the third farm. The possibility of mycoplasma infections in dairy cattle being transmitted from contaminated bedding or other environmental sources should be investigated further.

Résumé

Mycoplasma bovis est un organisme pathogène important des bovins, causant chez ces animaux la mammite, la pneumonie, l'arthrite et la métrite. Les infections par les mycoplasmes sont considérées contagieuses, la transmission d'un bovin à l'autre se faisant par l'intermédiaire d'un pis affecté par la mammite, lors de la tétée, ou par voie respiratoire. Dans le cadre du suivi d'une étude antérieure sur la prévalence des mycoplasmes, la présente recherche visait à évaluer la possibilité que les mycoplasmes soient présents dans l'environnement immédiat de la vache, dans les fermes laitières affectées par des mammites à mycoplasmes. À la suite d'un déclenchement de mammite causée par des espèces de mycoplasmes, nous avons mis en culture un échantillon de litière de sable prélevé dans l'aire de logement des vaches en lactation, et cet échantillon s'est avéré positif (c'est-à-dire contenant des mycoplasmes). Dans la même ferme, un échantillonnage subséquent dans 20 logettes sur litière de sable a révélé que 14 d'entre elles étaient positives pour les Mycoplasma spp. Nous avons ensuite isolé des mycoplasmes de la litière de deux autres fermes aux prises avec la mammite à mycoplasmes. Tous les échantillons positifs provenaient de litière de sable, à l'exception d'un échantillon provenant d'une logette sur paille. La présence de M. bovis fut confirmée par test de PCR dans les échantillons de deux des trois fermes (ce test n'ayant pas été effectué sur les échantillons de la troisième ferme). Cette étude souligne le besoin d'enquêter de façon plus approfondie sur la possibilité de la transmission des infections par les mycoplasmes chez les bovins laitiers à partir de la litière contaminée ou d'autres sources environnementales à la ferme.

Introduction

Infections with *Mycoplasma* spp, most commonly caused by *M. bovis*, constitute an important disease complex of dairy cattle. *Mycoplasma* spp affect all ages of cattle, and can cause metritis, arthritis, mastitis, pneumonia, agalactia, septicemia, and death of cattle.[3,10] Special laboratory methods are required for diagnosis because standard microbial cultures of milk samples do not isolate *Mycoplasma* spp.[6,7,12]

A previously reported survey of Utah dairy herds found 16/222 (7%) positive for *Mycoplasma* spp in bulk-tank milk.[13] During the follow-up phase of that prevalence study, we discovered an outbreak of clinical mastitis caused by mycoplasma on a 4,500-cow dairy farm that was associated with the isolation of *M. bovis* from recycled bedding sand and from freestalls bedded with sand. On other farms that also had mycoplasma in cows, investigation of possible mycoplasmas in bedding samples was conducted.

Materials and Methods

Eight Utah dairy farms previously diagnosed with mycoplasmal mastitis[13] were visited in this follow-up evaluation focusing on possible mycoplasma in the environment. Interest was stimulated when one of the consulting veterinarians for Dairy 1 contacted the authors regarding a mycoplasma mastitis outbreak on the farm approximately four months after the surveillance

project, and also related that they had cultured *Mycoplasma* spp from a recycled bedding sand sample.

Dairy 1 milked approximately 4,500 Holstein cows in dry lot and freestall housing, and experienced an outbreak of clinical mastitis (CM) caused by *Mycoplasma* spp. The endemic rate of CM cases from which *Mycoplasma* spp were isolated was three per month; during the outbreak there were 35 mycoplasmal CM cases per month (aseptic milk samples from all CM cases were cultured from this herd by the consulting veterinarian). Bedding sand was reused after going through a recycling, manure separation and water washing process on the farm. The sand was then stored in large, elongated piles. Samples of bedding sand and recycled sand were collected by the consulting veterinarians for Dairy 1, and cultured for mycoplasmas during the outbreak. Mycoplasma culture was performed using methods described below.

Composite bedding samples were then collected by the authors from the freestalls of Dairies 2 through 8, which were also farms where mycoplasma mastitis was confirmed in adult cows as follow-up to the mycoplasma prevalence study. Approximately 3 grams of material from the back one-third of six to 10 stalls per pen was collected into ziplock plastic bags. From hospital pens, one sample was collected from each of six different areas of the pen. Samples were cultured as described below. Real-time PCR testing was performed on one isolate from Dairy 1 and one from Dairy 8 at the Utah Veterinary Diagnostic Laboratory (UVDL) in Logan, UT.

Mycoplasma bedding culture methods

Bedding samples were shipped within 24 hours to the laboratory by courier. The on-farm monitoring at Dairy 1 used a milk quality laboratory in El Paso, TX. For monitoring bedding samples from the other seven farms, a milk quality laboratory in Greeley, CO was used. The samples were cultured using one gram of bedding diluted in 1000 ml of sterile water for an initial 1,000-fold dilution. Then three more 1:10 dilutions were made, resulting in $1:10^4$, $1:10^5$, $1:10^6$ dilutions. Mycoplasma culture was performed on Modified Hayflick medium incubated at 98.6°F (37°C) in a 10% CO_2 incubator using accepted methods.[5] Plates were read over 10 days, typically seven and 10 days after inoculation, and the number of colonies was counted. Final results were transmitted electronically to the UVDL.

Prolonged refrigeration experiments

Four bedding pile samples from Dairy 1 were refrigerated at 39.2°F (4°C) for 80 days. Samples were then cultured for *Mycoplasma* spp to evaluate survival of the organisms.

PCR protocol for mycoplasma detection

Two 1-gram aliquots of bedding sand were washed with phosphate buffered saline (PBS) and supernatant was collected. Two other 100 mg aliquots were placed in 5 ml of PPLO broth and incubated for 48 hours. DNA was extracted from 150 µl PPLO broth culture and from the PBS wash solution using a commercial extraction kit for tissues and fluids.[a] Briefly, the sample was mixed with 180 µl of tissue lysis buffer and 20 µl of proteinase K and incubated at 131°F (55°C) for two hours. After incubation, 200 µl of AL (Qiagen) buffer were added and the mixture was incubated for 10 minutes at 158°F (70°C). After addition of 200 µl ethanol, the mixture was transferred to the spin column containing a silica gel membrane that binds DNA based on the charge and pKa. The column was sequentially washed with 500 µl of each of the washing buffers and centrifuged at 5.9 X g for one minute. Finally, the DNA was eluted from the column by washing the column twice with 100 µl of AE (Qiagen) buffer.

A nested protocol developed by Baird[1] was used to process the extracted DNA per the standard protocol at the UVDL. The reactions were carried out on a thermocycler.[b] The PCR products were analyzed with a capillary electrophoresis platform[c] or by standard electrophoresis with a 2% gel run at 100 V for 1 hour and 20 minutes to confirm the detection of *M. bovis*. Confirmation was by DNA sequencing of the amplicon on a Sanger DNA sequencing platform[d] at the Center for Integrated Biosystems, Utah State University.

Results

After the first recycled bedding sand sample from Dairy 1 was found mycoplasma-positive, sand samples were cultured from 20 cow pens on the farm two months later. The owners of the 4,500 lactating cow herd were aware that they had mycoplasma before the surveillance project, and had been culturing milk from clinical mastitis cases and post-calving cows for more than one year; all cows identified with mycoplasma were culled. The mycoplasma clinical mastitis outbreak described earlier was still ongoing. Some pens contained freestalls and some were dry lots. *Mycoplasma* spp were isolated from 14/20 pen samples (70%) (Table 1). Colony forming units (cfu) per gram of bedding were not determined.

During the next three to four months, bedding samples from Dairies 2 through 8, which had also had mycoplasma detected in milk samples of cows, were tested for mycoplasmas. Used bedding sand from one pen on Dairy 3 was positive for *Mycoplasma* spp, 1,200 cfu/g (Table 1). Dairy 3 milked approximately 400 lactating cows, housed in outdoor freestalls. The owners were not aware of mycoplasma mastitis in the herd before the surveillance project. Following recent culture of the entire lactating herd, four cows had been found with *Mycoplasma* spp, and three had been culled. Therefore, one cow in the herd

was known to have mycoplasma mastitis at the time the positive bedding sand was found. The owners stated that because the remaining positive cow had never had clinical mastitis, they suspected that she was not truly infected and therefore their herd had become free of mycoplasma mastitis. However, the finding of mycoplasma in bedding convinced them that "it must still be in the herd".

Used bedding sand from one pen on Dairy 8 was positive for *Mycoplasma* spp, 72,000 cfu/g (Table 1). Dairy 8 milked approximately 2,800 lactating cows, housed in outdoor freestalls. The farm owners were aware that they had mycoplasma before the surveillance project, and suspected that they had previously introduced mycoplasma mastitis into the herd when they bought non-lactating replacement heifers. The precise number of cows diagnosed with mycoplasma mastitis as part of their ongoing culture program could not be determined, but was stated by the owners to be more than a few cows.

Table 1. Results of bedding cultures for mycoplasma from eight Utah dairy farms that previously had positive bulk-tank cultures for *Mycoplasma* spp.

Date	Dairy	Counts cfu/gm	Bedding type
12/2007	Dairy 1	positive	sand
3/2008	Dairy 1 14/20 pens	positive[1]	sand
6/19/2008	Dairy 2	neg.	sand
7/3/2008	Dairy 3 hospital	neg.	sand
7/3/2008	Dairy 3 pen 2	1,200	sand
7/3/2008	Dairy 4	neg.	straw
7/3/2008	Dairy 5	neg.	dried manure
7/3/2008	Dairy 6	neg.	sand
7/3/2008	Dairy 7	neg.	sand
7/3/2008	Dairy 8 pen 3	72,000	sand
8/28/2008	Dairy 1 pile A	8,000	sand
8/28/2008	Dairy 1pile B	8,000	sand
8/28/2008	Dairy 1 pile C	neg.	sand
8/28/2008	Dairy 1 pile D	neg.	sand
10/18/2008	Dairy 3 hospital	neg.	sand
10/18/2008	Dairy 3 pen 2	neg.	sand
10/18/2008	Dairy 1 pile E, deep	34,200	sand
10/18/2008	Dairy 1 pile E, superficial	neg.	sand
10/18/2008	Dairy 1 pile F, deep	neg.	sand
10/18/2008	Dairy 1 pile F, superficial	neg.	sand
10/18/2008	Dairy 1 pile G, deep	neg.	sand
10/18/2008	Dairy 1 pile G, superficial	neg.	sand
10/18/2008	Dairy 1 pile H, deep	neg.	sand
10/18/2008	Dairy 1 pile H, superficial	neg.	sand
10/18/2008	Dairy 1 pile I, deep	neg.	sand
10/18/2008	Dairy 1 pile I, superficial	neg.	sand
10/21/2008	Dairy 8 pen 1	560,000	sand
10/21/2008	Dairy 8 pen 2	4,200	sand
10/21/2008	Dairy 8 pen 12	30,000	sand
10/21/2008	Dairy 8 pen 15	5,600	sand
10/21/2008	Dairy 8 pen 19	6,000	straw
Samples collected 9/28/08 and refrigerated 39.2°F (4°C) for 80 days			
12/17/2008	Dairy 1 pile J	200,000	sand
12/17/2008	Dairy 1 pile K	62,000	sand
12/17/2008	Dairy 1 pile L	neg.	sand
12/17/2008	Dairy 1 pile M	neg.	sand

[1]The other six pens sampled at the same time were negative for *Mycoplasma*.

Two and three months later, 14 samples from nine piles of reused bedding sand were collected from Dairy 1. Surface sand samples from two piles were mycoplasma-positive (8,000 cfu/g) and one sand sample from deeper in another pile was also positive (34,200 cfu/g) (Table 1). The owners commented that some of the positive sand had been stored in a pile for more than one year after it had last been used in stalls, and had gone through the manure separation and water washing process.

Three months following the first sample collection from Dairy 8, bedding samples were collected from five outdoor freestall pens on the same farm; all were positive for *Mycoplasma* spp. The cfu/g of mycoplasma ranged from 4,200 to 560,000 (Table 1).

Mycoplasma spp were cultured from two of the four prolonged refrigeration experiment sand samples after 80 days at 39.2°F (4°C); 62,000 and 200,000 cfu/g (Table 1). In total, bedding samples (n=27) from three dairy farms with mycoplasma confirmed in adult cows as part of the follow up to the surveillance project, Dairies 1, 3, and 8 cultured positive for *Mycoplasma* spp. For one sample each from two dairies (1 and 8), the presence of *M. bovis* was verified with PCR; amplicon sequencing of both isolates showed 99% homology with *M. bovis*.

Most of the positive bedding samples (26/27; 96%) were sand bedding; the only exception was the identification of mycoplasmas in the straw bedding of one pen on Dairy 8; that farm also had four sand-bedded pens that were mycoplasma-positive.

Discussion

Outbreaks of mycoplasma mastitis and pneumonia have been associated with the introduction of new and presumably infected animals into dairy herds, as well as the recrudescence of infection in asymptomatic carriers associated with the physiologic stress of calving. Recognized modes of transmission are direct cow-to-cow by inhalation and respiratory secretions, and through contaminated milking equipment.[4,5,8,14]

Mycoplasma has been reported in dry lots and cooling ponds previously.[2,11] Survivability of various *Mycoplasma* spp in liquid media and on several farm-related substrates has also been investigated[9] and the results indicated that some species, *M. bovis*, *M. bovirhinis*, *M. arginini*, remained viable for 50 to more than 100 days in liquid media at 39.2°F (4°C) but only remained viable for seven to 28 days at outdoor temperatures on paper discs. However, there are no previous reports of *M. bovis* isolation from bedding on farms that also had *Mycoplasma* spp mastitis in cows; mycoplasma was found in many sand samples and one straw bedding sample from such farms in this study.

Some piles of recycled bedding sand were stored outside for more than one year before mycoplasmas were isolated. One herd owner only perceived the possibility that mycoplasma mastitis was still in his herd, despite keeping a known positive cow, after a bedding sample was found mycoplasma-positive.

Further evaluation of mycoplasmas in bedding and other environmental sources on dairy farms is needed. It is not certain whether mycoplasmas can infect dairy cows from the environment; future research should examine the potential for transmission of *M. bovis* and other mycoplasmas to dairy cattle in this way.

Endnotes

[a]Qiagen, Valencia, CA
[b]Eppendorf Gradient MasterCycler, Westbury, NY
[c]2100 Bioanalyzer, Agilent, Santa Clara, CA
[d]3730 DNA Analyzer, Applied Biosystems, Foster City, CA

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