Survival and replication of *Mycoplasma* species in recycled bedding sand and association with mastitis on dairy farms in Utah

A. Justice-Allen,^{*1} J. Trujillo,^{*} R. Corbett,[†] R. Harding,[‡] G. Goodell,[§] and D. Wilson^{*}

^{*}Department of Animal, Dairy and Veterinary Sciences, Utah Veterinary Diagnostic Laboratory, Utah State University, Logan 84321

[†]Robert Corbett, DVM, Spring City, UT 84662

[‡]Robert Harding, DVM, Spanish Fork, UT 84660

[§]The Dairy Authority, Greeley, CO 80634

ABSTRACT

Mycoplasma spp., usually *Mycoplasma bovis*, are important bovine pathogens that can cause mastitis, metritis, pneumonia, and arthritis. The currently documented routes of transmission of *Mycoplasma* spp. are through contaminated milking equipment and by direct animal contact. The existence of environmental sources for *Mycoplasma* spp. and their role in transmission and clinical disease is poorly characterized. *Mycoplasma* spp. (confirmed as *M. bovis* in 2 of 4 samples tested using PCR) was found in recycled bedding sand originating from a dairy experiencing an outbreak of clinical mycoplasma mastitis. *Mycoplasma* spp. were subsequently found in bedding sand from 2 other dairies whose bulk-tank milk was mycoplasma-positive. The association between the occurrence of *Mycoplasma* spp. in recycled bedding sand and mycoplasma mastitis in cows was further investigated using a pile of recycled sand from dairy 1. Study objectives included the determination of factors associated with the concentration of *Mycoplasma* spp. in recycled bedding sand and the duration of survival of mycoplasmas in the sand. We also evaluated the efficacy of 2 disinfectants at 2 different concentrations each for the elimination of *Mycoplasma* spp. from contaminated sand. *Mycoplasma* spp. survived in the sand pile for 8 mo. The concentration of *Mycoplasma* spp. within the sand pile was directly related to temperature and precipitation. It was also positively associated with the growth of gram-negative microorganisms, suggesting the possibility of the formation of a biofilm. Ideal temperatures for replication of *Mycoplasma* spp. occurred between 15 and 20°C. Moisture in the sand and movement of the sand pile also appeared to play a role in replication of mycoplasmas. We found that 0.5% sodium hypochlorite or 2% chlorhexidine were efficacious in eliminating *Mycoplasma* spp. from contaminated bedding sand. Recycled bedding sand could be an

environmental source of *Mycoplasma* spp., including *M. bovis*, infections in dairy cows. Future studies should investigate the contribution of this environmental source to the epidemiology of mycoplasma infections in dairy cattle.

Key words: mycoplasma, environment, mastitis, bovine

INTRODUCTION

Mycoplasma spp., typically *Mycoplasma bovis*, is an important disease in dairy cattle. Infection with *Mycoplasma* spp. can cause mastitis, arthritis, metritis, pneumonia, septicemia, and death of cattle (Pfutzner and Sachse, 1996; Wilson et al., 2007, 2009). Standard microbial cultures of milk samples do not isolate *Mycoplasma* spp.; special methods are necessary. Therefore, the presenting history associated with mycoplasma mastitis often includes a frustrating inability to determine the causative agent after clinical signs appear because of repeated negative results on standard microbial cultures of milk samples (González et al., 1992; Wilson et al., 2007). Culled or dead cows, lameness, respiratory disease in calves or adult cattle, and clinical mastitis that does not respond well to treatment can cause considerable financial loss (Pfutzner and Sachse, 1996). Per case, mycoplasmal infections cause some of the largest milk production losses among all mastitis pathogens (Wilson et al., 1997). Mycoplasma infections are contagious diseases and are primarily transmitted by milk from infected cows that contaminates milking equipment and by direct contact with respiratory secretions (Jasper, 1977; González et al., 1993; Ghazaei, 2006).

During the follow-up phase of a statewide prevalence study of mycoplasma mastitis in Utah, we discovered an outbreak of clinical mycoplasma mastitis on a dairy farm of 4,500 cows. This outbreak was associated with the isolation of *Mycoplasma* spp. from stored recycled bedding sand and from freestall bedding. An earlier reported outbreak of mycoplasma mastitis in a Florida dairy herd was associated with a mycoplasma-positive

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¹Corresponding author: anne.justice-allen@usu.edu

sandlot that was used for calving and to house mastitic cows (Bray et al., 1997). Additionally, seasonal variation of mycoplasma mastitis has been reported (González et al., 1992; Bray et al., 1997; González and Wilson, 2003); this variation could partially be attributed to seasonal cow stress, but environmental sources may play a role (Bray et al., 1997). Thus, the existence of environmental sources for *Mycoplasma* spp., including *M. bovis*, and their role in transmission and clinical disease needs further investigation.

The objectives of this study were to evaluate the concentration of *Mycoplasma* spp. in recycled bedding sand, investigate factors associated with the survival of *Mycoplasma* spp. in recycled bedding sand, and determine whether recycled bedding sand can serve as an environmental source of mycoplasmal infections of dairy cows. We also evaluated 2 methods of elimination of *Mycoplasma* spp. from contaminated bedding sand. Confirmation of an environmental source for *Mycoplasma* spp. in recycled bedding sand and its association with mycoplasma-related clinical disease raises possibilities for further investigation of mycoplasma disease in dairy cattle and could affect management strategies.

MATERIALS AND METHODS

Study Dairies

Mycoplasma-positive dairy herds were identified in a previously performed statewide prevalence study in Utah (Wilson et al., 2009). During the follow-up portion of that study, dairy 1 experienced an outbreak of clinical mastitis (CM) caused by *Mycoplasma* spp., of which 2 isolates were found by PCR to be *M. bovis*, from bedding sand and piles of recycled bedding sand that was designated to be reused. Dairy 1 had approximately 4,500 milking cows housed on dry lots and freestalls bedded with recycled sand. The endemic rate of CM attributable to *Mycoplasma* spp. was approximately 3 cases/4,500 cows per month. During the outbreak, aseptic milk samples from all CM cases were cultured from this herd by the herd health veterinarians (authors RH and RC). During the CM outbreak, the rate of mycoplasma mastitis cases increased to approximately 35 cases/4,500 cows per month. At this dairy, the bedding sand was recycled by scraping it out of the alleys and into a flume where the manure was washed free. This was followed by a second washing step on a cement pad with a low slope. After drying, the sand was swept up and stored for several months in large, elongated piles (approximately 3.75 m tall and 3.75 m wide by 600 m long) before reuse.

Samples of bedding sand from freestalls and the piles of recycled sand were collected by the herd health veterinarians. Composite bedding sand samples were collected from 20 freestall housing pens by collecting approximately 3 g from the back third of every tenth stall. Samples were identified by date and either the pen number or pile (letter designation). Dairy 1 began using sand as the only bedding substrate in 2001. In the first 2 yr, the fresh cow pen was bedded with clean sand that was brought in as needed from the sand and gravel company. In 2004, the dairy began using recycled bedding in all pens and no new sand was imported except during 1 mo at the end of the CM outbreak described.

Dairies 2 through 8 also had *Mycoplasma* spp. detected in bulk-tank milk as part of the prevalence study (Wilson et al., 2009). Composite bedding samples were collected (by author DW) from the freestalls of those dairies by collecting approximately 3 g of material from the back third of 6 to 10 stalls per pen. From hospital pens, 1 sample was collected from each of 6 different areas of the pen. Samples were identified with the dairy number, date, and pen number. Conventional mycoplasma culture methods identified *Mycoplasma* spp. in the freestall bedding sand and recycled sand. Polymerase chain reaction was used to determine whether the organisms isolated were *M. bovis* in 4 samples from dairy 1. The protocols for each method are described below.

Study Pile of Recycled Bedding Sand

Mycoplasma-positive recycled bedding sand (18,000 kg) was transported from dairy 1 to Utah State University (USU) and stored in a pile (approximately 3 m in diameter and 2 m tall) outdoors, uncovered, on a hard asphalt surface away from animals or their waste. The outbreak of mycoplasma mastitis at the dairy was resolving when the sand was removed from the dairy. The sand pile was composite sampled on the surface (approximately 0–1 cm into the pile) and deep (approximately 15–18 cm into the pile) layers on a weekly basis for 4 mo, from the beginning of April to the beginning of August. Each composite sample was collected with a disposable spoon and consisted of subsamples containing half of a spoonful from each of 5 sites within the pile. After 3, 4, and 5.5 mo of study, single site samples were taken from very deep (approximately 50 cm) in the pile. For each sampling, surface and deep, a new plastic spoon was used. The superficial sand that was removed from the deep sites was replaced in the sampling site after the sample was removed. Previously sampled sites were easily identified and a new set of sampling sites were used for each collection. The temperature of the

pile at the surface (2 cm) and deep (18 cm) locations was measured with a standard digital soil thermometer at 1600 h on each sampling day.

After 4 mo, the pile was composite sampled every 14 to 28 d for the next 2 mo (August–October). After 6 mo (the beginning of October), the sand was moved by a construction crew seeking access to a job site. Most of the sand was stored in a dump truck bed and some was taken to a nearby storage site away from animals or their waste; the sand was returned after 24 h. The sand was moved again approximately 3 wk later (Oct. 28) when it was relocated to another outdoor site, where it was stored uncovered, on cement, for use in another study. Weekly sampling resumed and continued for 7 wk.

Temperature and Precipitation Records for Logan, Utah

Weather data (daily highs and lows and daily precipitation in inches) were obtained from the USU Climate Center (<http://climate.usurf.usu.edu/products/data.php>) for the weather station closest to the sand pile (41°44'26"N, 111°48'37"W, elevation = 1,460 m). This climate center was less than 2 miles from the storage sites of the sand pile.

Sand Composition Testing

Sand nitrogen and carbon contents were determined by the USU Analytical Laboratory (USUAL). A composite sample of approximately 1 kg of sand was collected from the recycled bedding sand pile 1 mo after the sand arrived at USU. Additionally, a sample of unused sand from the source sand pit and composite samples of stored recycled bedding sand from dairy 1 were tested for total nitrogen, total carbon, and organic content.

Bedding Culture: From Dairies and from the Pile of Recycled Bedding Sand

Bedding samples were collected into new, resealable plastic bags. When freestalls were being sampled, 6 to 10 stalls were sampled from each pen; samples were taken from the back third of every tenth stall. Samples were held at 4°C and shipped within 24 h in padded envelopes to The Dairy Authority Laboratory (Greeley, CO) by priority mail (2-d delivery). Samples were cultured using 1 g of bedding diluted in 1,000 mL of sterile water to make an initial 1,000-fold dilution. Three additional 1:10 dilutions were made, resulting in 1:10⁴, 1:10⁵, and 1:10⁶ dilutions. For all dilutions, 50 µL

of sample was plated and counted to determine colony-forming units per gram. Aerobic culture was performed using MacConkey, modified Edward's agar (A70, Hardy Diagnostics, Santa Maria, CA), and mannitol salt agars for 48 h at 37°C. Plates were examined at 24 and 48 h and the growth was recorded. Mycoplasma culture was performed on modified Hayflick medium (G102, Hardy Diagnostics) using the same dilutions with 10% CO₂ and 80% humidity for 10 d. Plates were examined at 3, 7, and 10 d under 2× magnification with confirmation at 4× magnification, and the number of colonies morphologically consistent with *Mycoplasma* spp. were counted. Mycoplasma plates that were overgrown with other types of bacteria were defined as contaminated.

Controlled Experiments with Recycled Bedding Sand

Temperature Trials. Bedding samples were collected from deep in the pile and incubated at –20, 4, 20, 36, and 60°C in closed but not sealed Petri dishes for 24, 48, and 72 h. At 36°C, an additional trial was run by adding 250 µL of water at 24 and 48 h of incubation to keep the sample hydrated. An additional trial consisted of prolonged incubation for 117 d at 4°C of 4 recycled bedding sand samples (samples A, B, C, and D) from dairy 1. After incubation for the allotted time, the samples were sent overnight to The Dairy Authority Laboratory on frozen cold packs for mycoplasma culture and determination of colony-forming units per gram as described above. *Mycoplasma* spp. colony-forming units per gram were compared with those from the same samples before incubation.

Chemical Disinfection. A large sample was collected from deep in the pile after 2 mo and held at 4°C for 3 d. Sample fractions (2 g) of the sand were placed in 14-mL conical tubes and mixed with one of the following: 4 mL of 2% chlorhexidine gluconate solution (Durvet, Blue Springs, MO); 0.5% chlorhexidine gluconate (1:4 dilution of 2% solution); 1:10 dilution of household bleach (Clorox, Oakland, CA) and 0.5% sodium hypochlorite; or 1:30 dilution of household bleach (Clorox) and 0.17% sodium hypochlorite. Sterile deionized water was used as a control. The sand and disinfectant mixtures were allowed to stand undisturbed for 30 min at room temperature. The chlorhexidine or sodium hypochlorite was removed by decanting the liquid, adding 8 mL of sterile deionized water, mixing, settling, and decanting the liquid. The washing procedure was repeated 3 times. The treated samples (those treated with disinfectant) and the control samples were shipped on frozen cold packs to The Dairy Authority Laboratory for mycoplasma culture and determination of colony-forming units per gram of sample.

Conventional PCR Protocol

Sample Preparation. Sample preparation, DNA extraction, PCR and post-PCR processing were performed at the Utah Veterinary Diagnostic Laboratory. Two 1-g volumes of bedding sand were washed with PBS and the supernatant was collected. Additionally, two 100-mg volumes were placed in 5 mL of PPLO broth and incubated for 24 to 48 h at 37°C in 8% CO₂.

DNA Extraction. Following preparation, DNA was extracted from 150 µL of PPLO broth culture and from the PBS wash solution with a commercial extraction kit for tissues and fluids (Qiagen, Valencia, CA). Briefly, following the manufacturer's protocol, the sample was mixed with 180 µL of tissue lysis buffer and 20 µL of proteinase K (Qiagen) and incubated at 55°C for 2 h. After incubation, 200 µL of AL buffer (Qiagen) was added and the mixture was incubated for 10 min at 70°C. After the addition of 200 µL of ethanol, the mixture was transferred to the DNA binding spin column. The column was sequentially washed with 500 µL of each of the wash buffers (Qiagen) and centrifuged at 5,900 × *g* for 1 min. Finally, the DNA was eluted from the column by the addition of 100 µL of AE buffer (Qiagen) and centrifugation. Elution was performed twice.

Conventional PCR. Two conventional PCR protocols were used to detect *Mycoplasma* spp. or *M. bovis* DNA in the extracted DNA (Lanqing Li, Alabama State Veterinary Diagnostic Laboratory, Auburn, AL; personal communication). The *M. bovis*-specific PCR master mix consisted of 35 µL of RNase-free water, 5 µL of 10× PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 µL of MgCl₂ (50 mM), 1.0 µL of deoxyribonucleotide triphosphate (10 mM), 1 µL each of the forward and reverse primers (10 pmol/µL), and 2 U of Platinum Taq (Invitrogen). The forward *M. bovis*-specific primer (MYCO 5, Eurofins MWG Operon, Huntsville, AL) sequence was 5' TGATAGCAATATCATAGCGGC 3' and the reverse primer (MYCO 6, Eurofins MWG Operon) sequence was 5' GTAGCATCATTTTCCTATGCTAC 3'. The PCR reaction mix consisted of 45 µL of the PCR master mix and 5 µL of extracted DNA and produced 400 to 420 bp amplicon for identification of *M. bovis*. Each PCR assay included a positive *M. bovis* DNA control (*M. bovis*, ATCC # 25025) and a DNA-negative control (PCR-grade water); PCR was performed using a conventional thermocycler (Eppendorf Gradient MasterCycler, Westbury, NY). Cycling conditions for PCR included an initial denaturing step of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 5 min. The PCR products were analyzed with a capillary electrophoresis platform (2100 Bioanalyzer, Agilent,

Santa Clara, CA) or by standard electrophoresis with a 2% agarose gel run at 100 V for 1 h and 20 min for the identification of a PCR amplicon of equal size to the *M. bovis*-positive control.

The *Mycoplasma* spp. PCR protocol is a nested procedure. The flanking reaction uses a forward primer, MYCP 1 (5' ACACCATGGGAGCTGGTAAT 3', Eurofins MWG Operon) and a reverse primer, MYCP 2 (5' CTTCATCGACTTTCAGACCCAAGGCAT 3', Eurofins MWG Operon) with the same PCR master mix as the *M. bovis*-specific protocol. Each PCR reaction mix consisted of 5 µL of sample and 45 µL of master mix. The thermocycling conditions were as follows: 94°C for 45 s; 29 cycles of 94°C for 30 s, 55°C for 2 min, and 72°C for 1 min; and finishing with 72°C for 5 min and then a 4°C hold. The nested reaction used the forward nested primer, MYCO3 (5' GTTCTTTGAAACTGAAT 3', Eurofins MWG Operon) and the reverse nested primer, MYCO 4 (5' GCATCCACCAAAAACCTCT 3', Eurofins MWG Operon). The PCR master mix for the nested protocol was 37.5 µL of RNase-free water, 5 µL of 10X PCR reaction buffer (Invitrogen), 1.5 µL of MgCl₂ (50 mM), 0.5 µL of deoxyribonucleotide triphosphate (10mM), 2 µL each of forward and reverse primers (20 pmol/µL), and 2 U of Platinum Taq (Invitrogen). Each nested PCR reaction consisted of 49 µL of master mix and 1 µL of the flanking reaction product. The thermocycling conditions for this reaction were as follows: 94°C for 45 s; 33 cycles of 94°C for 30 s, 55°C for 2 min, and 72°C for 1 min; and finishing with 72°C for 5 min, followed by a 4°C hold. Positive and negative controls were included as described above. The PCR products were analyzed in the manner described above for the *M. bovis*-specific reaction.

RESULTS

Culture of Bedding from Study Dairies

Results of microbial culture of *Mycoplasma* spp. in bedding sand samples from the mycoplasma-positive dairies 1 through 8 are summarized in Table 1. Bedding sand from freestalls of dairies 1, 3, and 8 cultured positive for *Mycoplasma* spp. in 20 of 39 samples, 1 of 3 samples, and 6 of 6 samples, respectively (Table 1). Of the 2 samples of recycled bedding sand and the 2 samples of freestall bedding from dairy 1 tested with PCR, the presence of *Mycoplasma* spp. was verified in 2 of the recycled sand samples and 1 of the freestall bedding samples. *Mycoplasma bovis* was verified by PCR in 1 sand sample from freestall pens and 1 sample of recycled sand. The fourth sample was identified as positive for *Mycoplasma* spp. by microbial culture, but the PCR results were inconclusive. Except for the

Table 1. Detection of *Mycoplasma* spp. in bedding sand from 8 Utah dairies with mycoplasma-positive bulk tanks

Date	Dairy ¹	Count, ² cfu/g	Bedding type
Dec. 2007	Dairy 1	Positive	Sand
Mar. 2008	Dairy 1, 14/20 pens	Positive	Sand
Jun. 19, 2008	Dairy 2	Negative	Sand
Jul. 3, 2008	Dairy 3, hospital	Negative	Sand
Jul. 3, 2008	Dairy 3, pen 2	1,200	Sand
Jul. 3, 2008	Dairy 4	Negative	Straw
Jul. 3, 2008	Dairy 5	Negative	Dried manure
Jul. 3, 2008	Dairy 6	Negative	Straw
Jul. 3, 2008	Dairy 7	Negative	Straw
Jul. 3, 2008	Dairy 8, pen 3	72,000	Sand
Aug. 20, 2008	Dairy 1, pile A	8,000	Sand
Aug. 20, 2008	Dairy 1, pile B	8,000	Sand
Aug. 20, 2008	Dairy 1, pile C	Negative	Sand
Aug. 20, 2008	Dairy 1, pile D	Negative	Sand
Oct. 18, 2008	Dairy 3, hospital	Negative	Sand
Oct. 18, 2008	Dairy 3, pen 2	Negative	Sand
Oct. 18, 2008	Dairy 1, pile E; deep	34,200	Sand
Oct. 18, 2008	Dairy 1, piles E, F, G, H, I; superficial	Negative	Sand
Oct. 18, 2008	Dairy 1, piles F, G, H, I; deep	Negative	Sand
Oct. 21, 2008	Dairy 8, pen 1	560,000	Sand
Oct. 21, 2008	Dairy 8, pen 2	4,200	Sand
Oct. 21, 2008	Dairy 8, pen 12	30,000	Sand
Oct. 21, 2008	Dairy 8, pen 15	5,600	Sand
Oct. 21, 2008	Dairy 8, pen 19	6,000	Straw

¹Superficial samples were taken from the surface to a depth of 0 to 1 cm; deep samples were taken from a depth of 15 to 18 cm.

²Positive = colony-forming units per gram were not reported.

identification of *Mycoplasma* spp. in the straw bedding of pen 19 of dairy 8, all of the positive samples were sand or dirt bedding. *Mycoplasma* spp. colony-forming units per gram were not determined for dairy 1. The *Mycoplasma* spp. colony-forming units per gram for all other dairies ranged from 1.2×10^3 to 5.6×10^5 .

Dairy 1 Sand Pile Culture Results and Association with Temperature and Rainfall

***Mycoplasma* spp. Culture Results.** Results of microbial culture for *Mycoplasma* spp. in samples from the pile of recycled bedding sand collected from dairy 1 following an outbreak of clinical mycoplasma mastitis are summarized in Table 2. At the outset of the study, both the surface and deep samples were culture-positive, with 7.7×10^5 cfu/g and 2.13×10^6 cfu/g of sand, respectively (Table 2). The concentration of mycoplasmas in surface samples of the recycled sand pile rapidly decreased and the surface became mycoplasma culture-negative after 3 wk (Table 2). The samples from the deep portions of the pile became culture-negative on July 30, 2008 following apparent cyclic replication of *Mycoplasma* spp. deep within the pile, as evidenced by temporal fluctuations in colony-forming units per gram recovered from the pile that corresponded to changes in temperature and precipitation. Recovery of *Mycoplasma*

spp. from the sand pile appeared to be dependent on ambient temperature (Table 2). Not included in the table is a sample taken on July 8 from the very deep (50 cm) layer of the pile. This sample was positive at 60,000 cfu/g. Subsequent samples from this depth on August 5 and September 25 (also not included in the table) were negative.

Several factors, including ambient and sand pile temperatures, precipitation, and growth of other microorganisms in the pile, were investigated to determine their role in persistence and replication of *Mycoplasma* spp. within the pile of recycled bedding sand. Figure 1 is a graphical representation of the temperature changes within deep portions of the sand pile compared with the ambient maximum temperature over the duration of the study. Initially, the entire pile was visibly and palpably damp; as the spring and summer progressed, the surface became dry and crusted but the deep interior remained moist even when the deep samples collected were culture-negative for mycoplasma.

Figure 2 demonstrates the association of recovery of *Mycoplasma* spp. with temperature within the pile and precipitation. Initially, recovery of *Mycoplasma* spp. in the pile decreased quickly, which was followed by increasing and decreasing isolation of mycoplasma until the pile temperature stabilized at approximately 30°C and measurable precipitation ceased. *Mycoplasma* spp.

Table 2. *Mycoplasma* spp. (cfu/g) recovered from deep (15–18 cm) and superficial (0–1 cm) samples of recycled bedding sand, and environmental maximum temperature (Tmax)

Date	Deep	Superficial	Tmax, °C
Mar. 29, 2008	2,130,000	770,000	9.4
Apr. 6, 2008	990,000	720,000	6.1
Apr. 11, 2008	420,000	Negative	6.7
Apr. 15, 2008	182,000	Negative	23.3
Apr. 29, 2008	2,000	Negative	22.2
May 6, 2008	68,000	Negative	23.3
May 13, 2008	47,600	Negative	11.1
May 20, 2008	3,400	Negative	28.9
May 27, 2008	2,200	Negative	12.2
Jun. 3, 2008	7,800	Negative	23.3
Jun. 11, 2008	60,000	Negative	12.2
Jun. 17, 2008	4,000	Negative	30.0
Jun. 19, 2008	16,800	Negative	28.9
Jun. 30, 2008	2,000	Negative	33.3
Jul. 8, 2008	33,400	Negative	30.0
Jul. 15, 2008	Negative	Negative	30.6
Jul. 22, 2008	600	Negative	26.1
Jul. 30, 2008	Negative	Negative	33.9
Aug. 5, 2008	Negative	Negative	33.3
Aug. 20, 2008	Negative	Negative	31.7
Sep. 25, 2008	Negative	NR ¹	25
Oct. 6, 2008	Sand pile moved and replaced		
Oct. 10, 2008	14,200	1,000	14.4
Oct. 28, 2008	Sand moved to calf facility		
Oct. 30, 2008	1,400	Negative	20.6
Nov. 6, 2008	32,000	Negative	3.9
Nov. 12, 2008	600	Negative	8.3
Nov. 20, 2008	200	Negative	11.1
Nov. 28, 2008	Negative	Negative	11.1
Dec. 3, 2008	Negative	Negative	NR
Dec. 11, 2008	Negative	Negative	NR
Dec. 17, 2008	3,000	Negative	NR

¹NR = not recorded.

colony-forming units per gram increased 30-fold (from 2.0×10^3 to 6.0×10^4 cfu/g) and then decreased 3 times between May and July. With the first 2 spikes in *Mycoplasma* spp. colony-forming units per gram recovered from the pile, the pile temperature was between 15 and 20°C and there were recorded events of moderate precipitation. However, the last spike in *Mycoplasma* spp. concentration recovered from the pile during early summer was not associated with a recorded precipitation event and temperature in the pile was almost 30°C. Following this last spike in replication of *Mycoplasma* spp., the concentration of mycoplasma within the pile rapidly decreased as the temperature of the pile exceeded 30°C and this high temperature persisted. During the summer months, mycoplasmas were not recovered from the pile via culture. Measurable *Mycoplasma* spp. growth within the pile reoccurred when pile temperature decreased to 14°C and coincided with 2 of the largest precipitation events during the study. Precipitation on October 5 was followed by movement of the pile, during which the contents were rearranged and mixed. Subsequent samples collected were culture-positive for *Mycoplasma* spp. (1.4×10^4 cfu/g initially

deep within the pile) even though weekly samples during the previous 6 wk were culture-negative (Figure 2). When the pile was moved a second time to another outdoor location, there was an additional 20-fold increase in colony-forming units per gram (from 1.4×10^3 to 3.2×10^4 cfu/g) of *Mycoplasma* spp. recovered from the deep layer of the pile, which occurred 8 d following this movement.

Results demonstrate a positive association with precipitation and pile movement and increased replication and recovery of *Mycoplasma* spp. from the pile of recycled bedding sand, and a negative association between recovery of *Mycoplasma* spp. and ambient temperatures. After 6 wk of no recovery of *Mycoplasma* spp. from the pile, *Mycoplasma* spp. could be recovered from the pile after the favorable temperature and moisture conditions returned. Results indicate that there was long-term survival of *Mycoplasma* spp. in the recycled bedding sand, even during periods when none could be isolated by microbial culture.

Aerobic Culture Results. Figures 3 and 4 provide a comparison of growth patterns of *Mycoplasma* spp. to growth patterns of gram-negative microorganisms and

Figure 1. Temperature (°C) of the bedding sand at the deep level (T deep pile) compared with the ambient temperature (Tmax) of Logan, Utah.

Streptococcus spp. within the pile of recycled bedding sand over the duration of the study. The gram-negative microorganisms consisted primarily of *Pseudomonas* spp., and the reported colony-forming units per gram ranged from 6.8×10^5 in the deep layer of the bedding pile early in the year to undetectable in early August. Growth patterns of gram-negative microorganisms within the pile preceded or coincided with rises in *Mycoplasma* spp. colony-forming units per gram, but occurred at higher magnitude (Figure 3). *Streptococcus* spp. colony-forming units per gram ranged from 2.2×10^5 to undetectable, with the highest counts occurring

Figure 2. Recovery of *Mycoplasma* spp. within the recycled bedding sand pile and the cyclic growth of *Mycoplasma* spp. as it relates to precipitation and temperature. Colony-forming units per gram are reported as actual values $\times 10^{-3}$, deep temperature (T) is reported in °C, and precipitation (ppt.) is reported in inches $\times 10^2$. Asterisks indicate when the pile was moved (Oct. 7 and Oct. 28).

Figure 3. Association of the growth of *Mycoplasma* spp. and gram-negative microorganisms in recycled bedding sand. Colony-forming units per gram are reported as actual values $\times 10^{-3}$. Asterisks indicate when the pile was moved (Oct. 7 and Oct. 28).

early in the year and in July. The growth patterns of *Streptococcus* spp. within the pile for the duration of the study did not appear to be related to the growth of *Mycoplasma* spp. or other gram-negative microorganisms (Figure 4).

Sand Composition

The total nitrogen content was 0.03% and the total carbon content was 1.33% for the recycled bedding sand pile. The nitrogen and carbon contents were consistent with the presence of organic matter (Godden et al., 2008), which may play a role in biofilm formation in

Figure 4. Lack of a relationship between growth patterns of *Mycoplasma* spp. and *Streptococcus* spp. in recycled bedding sand. Colony-forming units per gram are reported as actual values $\times 10^{-3}$. Asterisks indicate when the pile was moved (Oct. 7 and Oct. 28).

Table 3. Percentage survival of *Mycoplasma* spp. at controlled constant temperatures in recycled bedding sand¹

Time	-20°C	4°C	20°C	36°C	36°C with H ₂ O ²	60°C
24 h	12.6	67.0	55.0	100	ND ³	Negative
48 h	6.7	6.7	32.5	Negative	7.2	Negative
72 h	2.5	4.8	9.1	Negative	37.5	Negative

¹Results are expressed as percentage survival, which was calculated as the number of organisms at time x divided by the number of organisms at time 0.

²At 24, 48, and 72 h of incubation, 250 µL of deionized tap water was added.

³ND = not done.

recycled bedding sand. The unused bedding sand had a total nitrogen content of 0.01% and a total carbon content of 1.48%. The amount of organic material in the unused bedding sand was 0.1%. Samples of recycled bedding sand taken from the stored piles at the dairy had a mean organic content of 2.1%, mean nitrogen content of 0.185%, and mean carbon content of 1.23%.

Temperature and Prolonged Refrigeration Effects on *Mycoplasma* spp. Growth and Survival

The controlled-temperature experiments were conducted to obtain more data relative to survival and optimal growth temperatures for *Mycoplasma* spp. in recycled bedding sand. Results of these experiments are provided in Tables 3 and 4. *Mycoplasma* spp. survived for 24 h at all controlled temperatures tested except 60°C (Table 3). For the short-term experiments, the highest percent survival rate occurred at 20°C, with 32.5% mycoplasma viable after 48 h and 9.1% mycoplasma viable after 72 h. At 36°C, the *Mycoplasma* spp. remained viable as long as the sand was kept damp by adding 250 µL of water every 24 h. After 72 h, the *Mycoplasma* spp. colony-forming units per gram in sand increased to 24,000 cfu/g from 4,600 cfu/g at 48 h, indicating possible replication at 36°C.

A prolonged refrigeration (4°C) survival experiment was carried out by storing 3-kg samples (n = 2) collected from dairy 1 for 117 d, moist, in sealed reclosable plastic bags (Table 4). Following prolonged incubation at 4°C, *Mycoplasma* spp. increased from 8,000 cfu/g to a mean 131,000 cfu/g. Replication of mycoplasma in recycled bedding sand occurred at temperatures from 4 to 36°C in the laboratory setting. These data are consistent with the effects of ambient temperature and temperature deep within the sand pile on *Mycoplasma* spp. growth patterns observed in the pile of recycled bedding sand that was kept outdoors.

Chemical Disinfection Experiments

The continuing survival of the organisms in the pile of recycled sand suggested that an environmental

source for *Mycoplasma* spp. could be established. We conducted experiments to evaluate the efficacy of 2 disinfectants in the reduction or elimination of mycoplasma in recycled bedding sand. Bleach (sodium hypochlorite) and chlorhexidine gluconate were chosen on the basis of availability, use in the dairy industry, and, for bleach, low cost. These 2 disinfectants were tested at 2 different concentrations. After incubation with the disinfectant, the sand was washed 3 times with deionized sterile water to reduce the disinfectant residue and to reduce inhibition of bacterial growth. Bedding sand treated with sterile water served as a control for these experiments.

The number of viable (cultivable) *Mycoplasma* spp. in recycled bedding sand was reduced in all treatment groups (Table 5). Treatment of contaminated sand with 0.5% chlorhexidine or 0.17% sodium hypochlorite resulted in the elimination of 97.3 and 93.3% of *Mycoplasma* spp., respectively. Treatment with higher concentrations of disinfectants, 2% chlorhexidine and 0.5% sodium hypochlorite, eliminated 100% of mycoplasma from contaminated bedding sand. Results demonstrate the initial efficacy of 2 common disinfectants in eliminating *Mycoplasma* spp. from contaminated recycled bedding sand.

DISCUSSION

Classically, recognized modes of transmission of mycoplasma-associated disease in dairy cattle include direct cow-to-cow transmission by inhalation and respiratory secretions and through contaminated milking

Table 4. Recovery of *Mycoplasma* spp. following prolonged refrigeration of bedding sand from dairy 1¹

Source pile	Initial count, cfu/g	End count, cfu/g
A	8,000	200,000
B	8,000	62,000
C	Negative	Negative
D	Negative	Negative

¹Initial collection date = Aug. 20, 2008; end collection date = Dec. 17, 2008.

Table 5. Concentration-dependent efficacy of 2 common chemical disinfectants in elimination of *Mycoplasma* spp. from contaminated recycled bedding sand¹

Item	Chlorhexidine gluconate		Na hypochlorite		Sterile water
	0.5%	2%	0.17% ²	0.5% ³	
Mean survival, cfu	900	Negative ⁴	2,200	Negative	33,000
Percentage reduction	97.3	100	93.3	100	Control

¹Data represent the mean of 2 replicates. Calculated percent reduction of *Mycoplasma* spp. as compared with the control (sterile water).

²1:30 bleach.

³1:10 bleach.

⁴Negative = *Mycoplasma* spp. not cultured.

equipment (Jasper, 1977; González and Wilson, 2003; Nicholas and Ayling, 2003). Others have found mycoplasma pathogens in cooling ponds and dry lots in association with mycoplasma disease in dairy herds, but the clinical significance of these environmental sources of this infectious agent remains undetermined (Bray et al., 1997; González and Wilson, 2003). Previous laboratory studies reported that *M. bovis* survived up to 236 d in manure and 23 d in drinking water at 23 to 28°C (González and Wilson, 2003). The finding of multiple mycoplasma-positive bedding samples associated with an outbreak of mycoplasma mastitis initiated the investigation reported here into the survival of mycoplasma in recycled bedding sand and factors associated with the persistence of this possible environmental source of *Mycoplasma* spp. Previous studies demonstrate that exposure of the teat ends to microorganisms in bedding can result in the development of mastitis (Kristula et al., 2005; Godden et al., 2008). The amount of mycoplasma required to induce mastitis is not well defined, but researchers have successfully induced mastitis by direct teat infusion of 70 cfu/g of *M. bovis* (Bennett and Jasper, 1980). In this study the amounts of *Mycoplasma* spp. recovered from the recycled bedding sand ranged from 10³ to 10⁶ cfu/g, suggesting that this environmental source may play a role in the epidemiology of mycoplasma-associated disease.

Moreover, the present results showed that *Mycoplasma* spp. remained viable in recycled bedding sand for a prolonged period of time (8 mo) and that the concentration of mycoplasma in the sand was associated with temperature, precipitation (moisture), and the growth of gram-negative microorganisms within the pile. There was reappearance of *Mycoplasma* spp. in the pile of recycled bedding sand that had been repeatedly culture-negative, and this reappearance of mycoplasma occurred following the return of favorable growth conditions and movement of the pile. Utilization of a pooled sampling method should have minimized the variability in the counts caused by location within the pile, so

increases of at least 10-fold can be interpreted as true increases in the numbers of organisms per gram. In addition, the smaller samples that were stored at 4 and 36°C also demonstrated similar increases. Therefore, it is likely that there was indeed growth of mycoplasma after the sand pile. A previous study of aerated and unaerated leachfield soil found that the active microbial population was 5 times higher in aerated soil (Amador et al., 2006).

Mycoplasma spp. possess a unique combination of traits: a genome that has undergone major reduction that includes loss of cell wall production; a resistance to some antibiotics as a result of the absence of a cell wall; reduced sensitivity to freezing but increased sensitivity to desiccation and chemical agents; and growth requirements for exogenous sources of sterols and lipids (Razin et al., 1998; González and Wilson, 2003; Walker, 2004). In our recycled sand pile study, we identified the occurrence of factors in recycled bedding sand over time that supported the establishment of an environmental source for *Mycoplasma* spp. Factors included sand pile temperature, precipitation (moisture), concurrent growth of gram-negative microorganisms, and low levels of organic material. Organic matter will support more bacterial growth than clean sand, and recycled bedding sand contains a low level of organic material (Godden et al., 2008). The variability of different bedding materials to support growth of microorganisms has been investigated (Kristula et al., 2005; LeJeune and Kauffman, 2005; Godden et al., 2008). Depending on the recycling process and storage conditions, recycled bedding sand will vary in the amount and composition of organic material that will provide support for bacterial growth (Kristula et al., 2005). The formation of a biofilm community that included mycoplasmas and gram-negative organisms could account for the survival and replication of mycoplasmas in what at first appears to be a growth factor-deficient environment. Biofilms are defined as a heterogeneous but highly organized community of microbial cells collected into microcolonies

embedded in extracellular polymeric substance matrix (Donlan, 2002; Halverson, 2005). Sand has been previously demonstrated as a matrix for biofilm development for *Escherichia coli* (Landini and Zehnder, 2002) and *Pseudomonas aeruginosa* (Holden et al., 2002; Robleto et al., 2003). A previous study (McAuliffe et al., 2006) found that many strains of *M. bovis* were capable of forming biofilms and, in a biofilm, *M. bovis* was more resistant to desiccation and temperature extremes. Our results support this observation and may explain why *Mycoplasma* spp., including *M. bovis*, can persist in a pile of recycled bedding sand over the hot, dry summer months.

In this study, the ideal temperature for survival and growth of *Mycoplasma* spp. in recycled bedding sand ranged from 15 to 20°C. *Mycoplasma* spp. replicated in the sand at 4°C when stored for 117 d and remained viable at temperature ranges from 4 to 27°C. Our results are consistent with previous investigations of the survivability of *Mycoplasma pneumoniae* (Wright et al., 1969). That study tested the viability of this organism in an aerosol at controlled temperatures; *M. pneumoniae* survived best at 15°C. Another study found that some species of mycoplasma, including *M. bovis*, *Mycoplasma bovirhinis*, and *Mycoplasma arginini*, remained viable from 50 to more than 100 d in liquid media at 4°C but remained viable for only 7 to 28 d at outdoor temperatures on paper discs (Nagatomo et al., 2001).

The present study demonstrated that *Mycoplasma* spp. occurred in high numbers in bedding sand from a dairy concurrently experiencing a clinical outbreak of mycoplasma mastitis and that an environmental source for *Mycoplasma* spp. can be established in recycled bedding sand. Therefore, we conducted experiments to evaluate the efficacy of 2 common disinfectants in the reduction or elimination of *Mycoplasma* spp. in recycled bedding sand. Reportedly, the advantage of using sand as a bedding material is the potential to remove or inactivate most of the microbial contamination through washing (Kristula et al., 2005; Godden et al., 2008); however, in our study, *Mycoplasma* spp., including some isolates confirmed by PCR as *M. bovis*, and other microorganisms could be recovered from washed (recycled) bedding sand. Following treatment with 2% chlorhexidine and 0.5% sodium hypochlorite, 100% of *Mycoplasma* spp. were eliminated from contaminated bedding sand, demonstrating the efficacy of 2 common disinfectants in eliminating mycoplasma or at least making it such that none could be isolated, thereby reducing the potential for the transmission of disease.

In summary, *Mycoplasma* spp. was discovered in high numbers in recycled bedding sand originating from a dairy experiencing an outbreak of clinical mycoplasma mastitis and was also found in bedding from 2 other

farms with mycoplasma-positive cows. *Mycoplasma* spp. survived in recycled bedding sand for 8 mo and replication was associated with temperature, precipitation, and replication of gram-negative microorganisms. Recycled bedding sand appeared to be an environmental source of *Mycoplasma* spp. Results warrant investigation of the contribution of biofilm formation in the establishment of this environmental source and the epidemiological role of this source of mycoplasma-associated disease. Chemical disinfection appeared to be effective, but practicality and cost-effectiveness require further study. Moisture appeared to contribute to the survival and replication of the organism in bedding sand. This is also an avenue for further investigation because one method of reducing the numbers of all bacteria in recycled bedding sand could be to improve drying by repeatedly turning the piles, at least in arid climates such as in the western United States.

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