

Effects of Temperature and Humidity on the Survival of Mycoplasma Organisms in Recycled Bedding Sand

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Abstract

Mycoplasma bovis, along with other species, is responsible for a number of disease syndromes in cattle which impact both the dairy and beef industries. An outbreak of mycoplasma mastitis in conjunction with a number of positive environmental samples from the farm initiated a study of the survival of mycoplasma organisms in recycled bedding sand. A pile of bedding sand was obtained from the affected dairy and brought to Utah State University, where it was stored outside. The pile was cultured for mycoplasmas and mastitis pathogens on a weekly basis for four months, and then sampled every two to four weeks for another four months. In addition, samples of the sand were subjected to controlled experiments in the laboratory involving temperature, humidity, and chemical disinfection. The results indicate that *M. bovis* can remain viable and detectable by culture in recycled bedding sand for eight months at the ambient temperatures found in Logan, Utah. The optimal temperature for survival is between 39°F (4°C) and 64°F (18°C). The numbers of *M. bovis* organisms (measured as colony forming units) in the sand increased at different times during the study, indicating replication. *M. bovis* reproduced in bedding sand at ambient maximum temperatures between 59°F and 68°F (15°C and 20°C). In the controlled experiment, mycoplasmas reproduced in the bedding sand at 97°F (36°C) when the sand was kept damp, but were not cultivable when it was allowed to dry out. Both 2% chlorhexidine and 0.5% hypochlorite were capable of disinfecting the bedding sand.

Résumé

Mycoplasma bovis, ainsi que d'autres espèces, causent de nombreux syndromes morbides chez les bovins, qui affectent l'industrie des bovins laitiers et de boucherie. Un déclenchement de mammite à mycoplasmes dans une ferme laitière, conjointement avec la découverte de plusieurs échantillons contaminés par les mycoplasmes dans l'environnement d'élevage de

cette ferme, nous a conduits à mener une étude sur la survie de cette bactérie dans le sable de litière recyclé. Un tas de sable de litière fut récolté de la ferme affectée puis entreposé à l'extérieur sur notre site de recherche à l'Université de l'État de l'Utah. Pendant quatre mois, nous avons vérifié la présence de mycoplasmes et d'autres organismes responsables de la mammite par la mise en culture d'échantillons de sable, une fois par semaine, puis toutes les deux à quatre semaines pendant quatre autres mois. Parallèlement, des échantillons de sable ont été soumis à des expériences contrôlées en laboratoire portant sur la température, l'humidité et la désinfection chimique. Les résultats indiquent que *M. bovis* peut rester en vie dans du sable de litière recyclé (et détectable par mise en culture) pendant huit mois à la température ambiante extérieure de Logan, en Utah. La température optimale de survie se situait entre 4° C (39° F) et 18° C (64° F). Le nombre d'organismes de *M. bovis* (évalué d'après le nombre d'unités formant colonies) retrouvés dans le sable a augmenté à différents moments durant l'étude, ce qui indique qu'il y a eu reproduction. *M. bovis* s'est reproduit dans le sable de litière à une température ambiante maximale allant de 15° C à 20° C (59° F à 68° F). Dans l'expérience contrôlée, les mycoplasmes se sont reproduits dans le sable de litière maintenu humide à une température de 36° C (97° F), mais n'ont pu être cultivés à partir d'échantillons de sable soumis à cette température qu'on avait préalablement laissé sécher. Des solutions de chlorhexidine à 2 % et d'hypochlorite à 0,5 % ont pu désinfecter le sable de litière.

Introduction

Mycoplasma spp, particularly *M. bovis*, cause a number of disease syndromes in cattle. Outbreaks of these diseases in beef and dairy calves and dairy cows cause significant losses to the industry.^{14,18} Infections caused by mycoplasmas have generally been considered contagious by direct means such as respiratory secretions, or indirectly by contaminated milking equipment.^{6,7,10} Outbreaks often occur a few weeks to months after the introduction of new animals; in mycoplasma-

positive herds, a latently infected individual will often shed the organisms in moderate numbers at times of physiological stress and infect herd mates.^{5,8} Because they lack a cell wall and require culture media that have added sterols, and nucleic acids often in the form of horse serum and yeast extract,¹⁷ the survival of mycoplasmas in the typical dairy or calf farm environment has not been considered a factor in disease transmission. However, mycoplasmas associated with mastitis have been found in cooling ponds and dry lots, but the significance in relation to the occurrence of mastitis in the herds was unknown.^{2,8} Researchers have examined the survival of mycoplasmas on various substrates, in liquids, and at different temperatures.⁸ The studies demonstrated that some species—*M. bovis*, *M. bovirhinis*, and *M. arginini*—remained viable from 50 to more than 100 days in liquid media at 39°F (4°C); at ambient outdoor temperatures on paper discs the viability decreased to seven to 28 days. An earlier study examined organism survival on a number of substrates; the mycoplasmas remained viable for 236 days in dried manure in the dark, but were viable for at most a few days on wood, glass, corn straw, and cotton stuff.⁸

During the follow-up phase of a study¹⁹ of the prevalence of mycoplasma in dairy farms in Utah, one of the positive dairies experienced an outbreak of mycoplasma mastitis. This farm had 4,500 cows housed in dry lot pens with free stalls. The free stalls were bedded with sand, which was reused after washing and separating the manure. The herd health veterinarians conducted an epidemiological study in order to determine the factors that led to the outbreak. During the investigation, they cultured the bedding in the free stalls and the recycled sand piles, as well as clinical cases. Fifteen of 20 environmental samples collected over a period of three months cultured positive for *Mycoplasma* spp; one sample was tested and confirmed positive for *M. bovis* with a conventional nested PCR method¹ in use at the Utah Veterinary Diagnostic Laboratory (UVDL), definitively identifying the mycoplasma present in the bedding sand. The occurrence of *Mycoplasma* spp in both the free stall bedding sand and the piles of stored sand that were allocated for reuse in conjunction with an outbreak of mycoplasma mastitis provided the impetus for further examination of the survival of mycoplasmas in recycled bedding sand.

Materials and Methods

Recycled bedding sand pile

Mycoplasma-positive bedding sand (40,000 lb; 18,182 kg) was transported from the affected dairy farm to Utah State University (USU) in April and stored outdoors. The sand pile was sampled on the surface (0.75 inches into the pile) and deep (6 to 7 inches into

the pile) on a weekly basis for four months (July 30). After four months, the pile was sampled every 14 to 28 days for the next two months. After six months (October 6), the sand was moved by a construction crew in order to access the roof of the building to which the pile was adjacent. Most of the sand was stored in a dump truck bed and some was taken to a nearby storage site. The sand was returned to the study site after 24 hours. The sand was moved again one month later, seven months after transport to USU, when it was taken to another outdoor location. Weekly sampling resumed again and continued for seven weeks until December 17. After 3, 4, and 5½ months of study, samples were taken from very deep (approximately 20 inches) in the pile. For each sampling level, surface and deep, a clean plastic spoon was used to sample five locations. The superficial sand that was removed from the deep sites was restored after the sample was removed. Previously sampled sites were easily identified and a new set of sampling sites was used for each sample collection date. The temperature of the pile at the surface (0.75 inches) and deep (7 inches) locations was also recorded at each sampling.

Bedding cultures

Bedding samples were collected into new ziplock plastic bags and shipped to the Dairy Authority (TDA, Greeley, CO) by courier. The samples were cultured using one gram of bedding diluted in 1000 ml of sterile water to make an initial 1,000-fold dilution. Then three more 1:10 dilutions were made, resulting in 1:10⁴, 1:10⁵, 1:10⁶ dilutions. Aerobic culture was performed using MacConkey, selective Strep, and Mannitol Salt agars for 48 hours at 98.6°F (37°C). Plates were examined at 24 and 48 hours, and the colony growth recorded as colony forming units (CFU). Mycoplasma culture was performed on modified Hayflick media using the same dilutions with 10% CO₂ and 80% humidity for 10 days. Plates were examined after seven and 10 days and the number of colonies were counted (CFU). Colonies were morphologically consistent with *M. bovis*.

Controlled experiments with recycled bedding sand

Temperature trials. Aliquots were taken from deep in the pile and incubated at -20°F, 39°F, 68°F, 97°F, and 140°F (-20°C, 4°C, 20°C, 36°C, and 60°C) in covered, but not sealed, petri dishes for 24, 48, and 72 hours. A large aliquot of sand was kept refrigerated for 80 days. In a second trial at 97°F (36°C), 0.25 ml of sterile deionized water was added to an approximately 1 gm sample of sand. If during the incubation period the sand appeared to be drying out, additional water was added. After incubation for the allotted time, the samples were sent to TDA on frozen cold packs for culture and colony count enumeration. Mycoplasmal colony counts were compared to those from the same samples before incubation.

Chemical disinfection. A large sample was collected from deep in the pile after two months and held at 39°F (4°C) for three days. Aliquots (approximately 2 gm) of the sand were placed in 14 ml conical tubes, and mixed with 4 ml of deionized sterile water (for a negative control), 0.5% chlorhexidine gluconate, 2% chlorhexidine gluconate, 1:30 dilution of household bleach (0.17% sodium hypochlorite), or 1:10 dilution of household bleach (0.5% sodium hypochlorite). The sand/disinfectant mixture was allowed to stand undisturbed for 30 minutes at room temperature. The disinfectant was removed by decanting the liquid, adding 8 ml of sterile deionized water, mixing, settling, and decanting the liquid. This washing procedure was repeated three times. The treated samples and an untreated, control sample were shipped on frozen cold packs to TDA for culture and CFU determination.

Weather records

Weather data (daily highs and lows) for the study period were downloaded from the Utah Climate Center at USU for the local weather station (lat. 41°44'26", long. -111°48'37", elev. 1460 meters).¹⁶

Results

Number of mycoplasmas per gram of sand and the temperature

At the outset of the study, both the surface and deep cultures were positive at greater than 10⁵ mycoplasma organisms per gram of sand (Figure 1). The surface cultures were negative after three weeks. The colony counts of the deep samples declined slowly over a period of 18 weeks, becoming repeatedly negative at the end of July. There were several times when the number of mycoplasmas per gram increased one log₁₀ from 10³ cfu/gm to 10⁴ cfu/gm; this corresponded to an environmental temperature between 59°F and 68°F (15°C and 20°C). The surface temperature of the pile fluctuated more dramatically than the interior (Figure 2), and subjectively, the surface of the pile was dry and crusted while the interior remained damp. Six months into the study the pile was picked up and moved for one day because of a construction project, which rearranged and mixed the contents. Even though six previous cultures over a period of six weeks were negative, the pile again cultured positive (1.4 X 10⁴ cfu/gm) for *Mycoplasma* spp and remained positive for a month after the pile was moved (Figure 1). This increase in numbers of mycoplasmas was repeated when the pile was moved a second time to another outdoor location.

Aerobic culture results. The results of the aerobic culture of the sand were reported as general categories of mastitis organisms (colony forming units per gram

of bedding). The coliforms were noted to be primarily *Pseudomonas* spp on a number of reports; the numbers ranged from 6.8 X 10⁵ in the deep layer of the bedding pile early in the year to undetectable in early August. Like the *Mycoplasma* spp, the numbers increased after the pile was moved and then remained at an average of 5.5 X 10⁵ for the remainder of the study. The numbers of coagulase-negative *Staphylococcus* spp ranged from

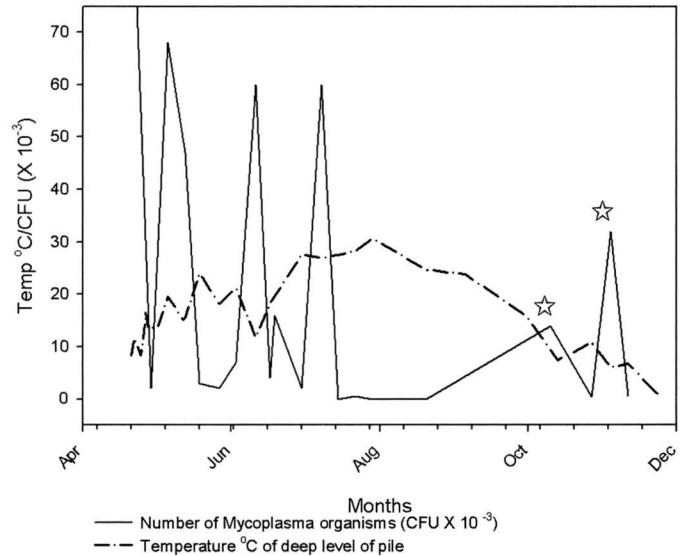


Figure 1. Number of mycoplasma cultured per gram (CFU X 10⁻³) of bedding from the deep layer of the pile compared to the temperature (°C) of the deep layer of the bedding pile. Bedding pile moved at ☆.

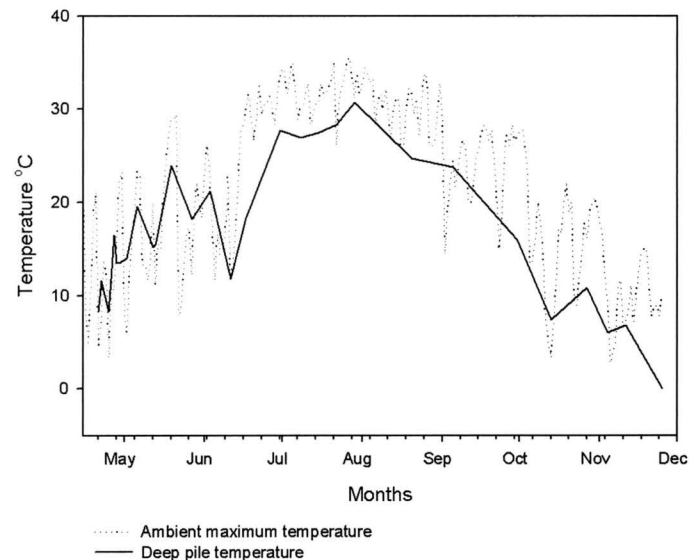


Figure 2. Ambient maximum temperature compared to temperature of the deep layer of the sand pile demonstrating a moderation of temperature change deep in the pile.

3.6 X 10⁶ early in the year to 2 X 10³ in early August in the samples from the deep layer of the pile. *Streptococcus* spp ranged from 2.2 X 10⁵ to undetectable, with the highest counts occurring early in the year and July.

Controlled temperature and chemical disinfection experiments

M. bovis in recycled bedding sand survived for 24 hours at all controlled temperatures tested except 140°F (60°C) (Table 1). The highest percent survival rate occurred at 68°F (20°C) with 32.5% still viable after 48 hours and 9.1% still viable after 72 hours. However, very low viability rates occur at 39°F (4°C) for as long as 80 days. At 97°F (36°C), the *M. bovis* remained viable as long as the sand was kept damp; after 72 hours, the number of organisms per gram increased.

The number of viable *M. bovis* in recycled bedding sand decreased with all of the disinfection protocols used (Table 2). With 2% chlorhexidine and 1:10 diluted bleach, no *M. bovis* organisms were cultured; however, weaker concentrations of each of the disinfectants did not kill all of the mycoplasmas in the sand.

Discussion

With the exception of an early study of *M. bovis* survival in manure and water, previous studies on the

survival of mycoplasmas in various environments found relatively short survival times of seven to 28 days at ambient environmental temperatures.^{3,8} The results reported here indicate that *M. bovis* can remain viable for a much longer period under conditions that occur on a typical dairy farm. Both the controlled experiment and the sand pile study suggested that *M. bovis* survived best between 39°F (4°C) and 64°F (18°C). Freezing temperatures and warm conditions (greater than 86°F) reduced survival. Another interesting finding was that *M. bovis* can reproduce in the bedding sand under the proper conditions. Both in the sand pile and in the controlled experiments, the number of mycoplasmas cultured per gram of bedding increased at times. In the bedding pile, the number of organisms went from undetectable to 10⁴ per gram, while in the controlled temperature experiment the number of organisms increased one log₁₀ in 24 hours.

When the pile was moved and mixed, aeration probably occurred and appeared to contribute to the growth of the coliforms and the mycoplasmas. We hypothesize that one explanation for the growth of mycoplasmas in sand is the formation of a biofilm. A biofilm is a heterogeneous collection of microbial cells organized into microcolonies and encased in an extracellular matrix.⁴ Sand has been used to study biofilm development by *Escherichia coli* and *Pseudomonas aeruginosa*.^{9,12,15}

Table 1. Percent survival of *M. bovis* in recycled bedding sand at controlled constant temperatures.

Time	- 4°F (-20°C)	39°F (4°C)	68°F (20°C)	97°F (36°C)	97°F (36°C) w/H2O	140°F (60°C)
24 hr.	12.6*	67.0	55.0	100	ND	neg.
48 hr.	6.7	6.7	32.5	neg.	7.2	neg.
72 hr.	2.5	4.8	9.1	neg.	37.5	neg.
90 days	ND	0.5	ND	ND	ND	ND

*Expressed as percent survival; ND—not done

Table 2. Effectiveness of chemical disinfection for mycoplasmas in recycled bedding sand subjected to 30 minutes of contact time with disinfectant.

Treatment	Sterile water	Chlorhexidine 0.5%	Chlorhexidine 2%	0.17% Na hypochlorite ^c	0.5% Na hypochlorite ^d
Replicate 1	64,000 ^a (100%)	1,600 (2.5%)	neg. ^b	4,400 (6.9%)	neg.
Replicate 2	2,000 (100%)	200 (10.0%)	neg.	neg.	neg.

^aColony forming units (% survival compared to original mycoplasma concentration)

^bneg. = No mycoplasmas isolated

^c1:30 bleach

^d1:10 bleach

A recent study tested the ability of several species of mycoplasma to form biofilms;¹³ *M. bovis* formed prolific biofilms on a glass substrate (broken coverslips). In addition, the study found that *M. bovis* organisms in a biofilm were more resistant to heat and desiccation. Biofilms in nature consist of complex communities in which different species play different roles in the community and both metabolic and structural relationships can be identified.¹⁶ The coliforms and other bacteria could be providing the nucleic acids and sterols that the mycoplasmas require for growth. The results presented in this study suggest that more research is needed on the role of biofilm formation in the farm environment and the role that other organisms may play in the development of biofilm communities.

M. bovis is capable of surviving in a typical farm environment, especially recycled bedding sand. It is uncertain whether the levels reported here are sufficient to cause infection of either the mammary gland or the respiratory tract, but the sand used in this research originated from a farm experiencing an outbreak of mycoplasma mastitis due to *M. bovis*. Future research should be directed toward investigating this route. In addition, chemical disinfection appears possible, but a method needs to be developed that would be practical for farm implementation.

Sand, because of its inorganic nature, has been perceived to be a cleaner bedding material because it often contains fewer organisms than organic materials, such as shavings or straw.¹¹ In addition, several types of manure separating and washing systems are being promoted to clean and recycle the bedding. One study of recycled sand found only minor increases in organic matter and nearly identical bacterial counts over several days of sampling.¹¹ The sand pile used in the present study had been subjected to washing on the farm but still contained moderate numbers of microbes, including mycoplasmas. In the laboratory, 0.5% hypochlorite and 2% chlorhexidine were capable of killing all of the *M. bovis* in the test sample. While the chlorhexidine is probably too expensive to be used for cleaning large quantities of sand, further investigation of hypochlorite as a practical disinfectant is needed. Perhaps the most practical method of reducing the number of organisms in the bedding would be to repeatedly spread and turn the pile, exposing more of it to temperature extremes and promoting drying.

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