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Case report – Utilizing formic acid to effectively eliminate *Mycoplasma bovis* in unpasteurized fresh raw milk

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Abstract

Calves fed milk infected with *Mycoplasma bovis* are at risk of developing mycoplasma-associated diseases such as pneumonia, otitis media, and arthritis. This study was conducted to determine if acidification of milk cultured positive for *M. bovis* would consistently result in complete elimination of the organism. Three consecutive daily milk samples were obtained from 8 cows with confirmed *M. bovis* mastitis. An aliquot of each milk sample was submitted for culture and enumeration of *M. bovis* as colony-forming units per milliliter (cfu/mL). Dilute (9.8%) formic acid was used to acidify the remainder of each sample, after which culture and enumeration of *M. bovis* was repeated. Prior to acidification, *M. bovis* was detected in all milk samples, but the levels present were highly variable both between cows on a given day and within cow between days. On day 1, the median was 418 cfu/mL of *M. bovis* (interquartile range (IQR) 25th and 75th percentile (125 to 1,445; range 15 to 3,640 cfu/mL)). Equivalent results for day 2 were median 155 cfu/mL (IQR 30 to 1,330; range 20 to 2,280 cfu/mL), whereas day 3 *M. bovis* levels were median 35 cfu/mL (IQR 27 to 230; range 15 to 440 cfu/mL). Each of the samples collected on days 1 to 3 was culture negative for *M. bovis* following acidification. Results from the current study demonstrate that formic acid is effective for consistently eliminating *M. bovis* from culture-positive milk.

Key words: milk, acidification, *Mycoplasma bovis*, formic acid

Résumé

Les veaux alimentés au lait contaminé par *Mycoplasma bovis* sont à risque de développer des maladies associées au mycoplasme telles que la pneumonie, l'otite moyenne, et l'arthrite. La présente étude visait à déterminer si l'acidification de lait positif pour *M. bovis* résulterait systématiquement en l'élimination complète de cet organisme. Trois échantillons consécutifs de lait ont été obtenus de 8 vaches atteintes de mammite à *M. bovis*. Un aliquot de chaque échantillon a été soumis à un test de culture et au dénombrement microbien de *M. bovis* en unité formant colonie par mililitre (cfu/mL). Le reste de chaque échantillon a ensuite été acidifié à l'aide d'acide formique diluée (9.8%) et également soumis à un test de culture et au dénombrement microbien *M. bovis*. Tous les échantillons de lait non-acidifiés ont été testés positifs pour *M. bovis*, mais les niveaux étaient hautement variables entre les vaches pour un jour donné, ainsi que pour une même vache sur différents jours. Au Jour 1, la médiane était 418 cfu/mL de *M. bovis* (écart interquartile (IQR) 25^{e} et 75^{e} percentiles (125 à 1,445; écart 15 à 3,640 cfu/mL)). Des résultats équivalents ont été obtenus au Jour 2 avec une médiane de 155 cfu/mL (IQR 30 à 1,330; écart 20 à 2,280 cfu/mL), tandis que les niveaux médians de *M. bovis* au Jour 3 ont été de 35 cfu/mL (IQR 27 à 230; écart 15 à 440 cfu/mL). Chacun des échantillons collectés aux Jours 1 à 3 sont ressortis négatifs en culture pour *M. bovis* après acidification. Les résultats de la présente étude démontrent l'efficacité systématique de l'acide formique à éliminer *M. bovis* du lait testé positif.

Introduction

Mycoplasma bovis, formerly *Mycoplasma agalactiae* subsp *bovis*, was first identified from a case of mastitis in 1961.[16] *Mycoplasma* spp have been incriminated in cases of mastitis, arthritis, pneumonia, otitis media, and inflammation of the urogenital tract in cattle.[15,18] Regional differences are seen in the herd-level prevalence of mycoplasma mastitis across the United States, and in 2007, USDA-APHIS reported a prevalence of 3.2% of dairy herds.[2] *M. bovis* is able to infect the bovine mammary gland at any point of the production cycle and may either result in subclinical, clinical, or chronic infections.[13] Classically, clinical signs of mycoplasma mastitis include infection of multiple quarters; a marked decrease in milk production; abnormal udder secretions which vary from thick, viscous material to watery with sandy or flaky sediments; and resistance to antibiotic therapy.[5,17] *Mycoplasma* spp associated with mastitis are categorized as contagious

pathogens and are indirectly transmitted between cows, primarily at milking time. Common fomites include hands of milkers, milking unit liners, teat-dip cups, and udder wash towels.[9] Once members of a herd become infected with *M. bovis*, the bacteria can be easily transmitted to uninfected cattle.[18]

Chronic asymptomatic infection with intermittent shedding is critical to the epidemiology of *M. bovis*, and especially its maintenance within a herd and exposure of naive populations.[12,18] Transmission is often delayed until an asymptomatic carrier sheds the organism, making it hard to identify the point source of infection during an outbreak.[23] Only 70 colony-forming units (cfu) of *M. bovis* are needed to pass through the teat canal of a susceptible cow to initiate infection.[4] Calves often acquire *M. bovis* infections by ingesting milk infected with the organism and by being in close contact with infected calves.[20] Pneumonia, otitis media, and arthritis are common sequelae for calves infected with *M. bovis*. Diagnosis of mycoplasma mastitis is most commonly made using microbiological procedures, namely direct culture of milk samples and identification of typical colony morhphology.[11,13]

In the dairy industry, the practice of acidifying waste milk as a means of reducing the bacterial pathogen load in the milk diet of calves is growing in popularity and may help limit disease transmission on farms.[1] The current study was conducted to determine if acidification of milk cultured positive for *M. bovis* would eliminate the organism. The objective of this case report is to describe the method of reducing the transfer of *Mycoplasma bovis* by acidifying waste milk with formic acid.

Materials and Methods

This study was performed with the cooperation of a 1500-cow dairy in southeast Pennsylvania. The farm is a regular client of the University of Pennsylvania Field Service at New Bolton Center. Owner consent was obtained, and the study was approved through the veterinary school's 'Privately Owned Animal Protocol' procedure. The diagnosis of *M. bovis*-associated infections in both adult cows (pneumonia, arthritis, and mastitis) and calves (pneumonia, arthritis, and otitis media) was confirmed by culture. Bulk-tank samples were also culture-positive for *M. bovis.*

Preparation of dilute formic acid

Pure (98%) formic acid[a] was diluted 1:10 (50 mL formic acid in 500 mL of water) to achieve a working solution concentration of 9.8%. The diluted acid solution was used to acidify the milk samples in this study, based on instructions outlined by Anderson.[1]

Collection and handling of M. bovis-infected milk samples

Routine surveillance of the dairy's bulk tank revealed the presence of *M. bovis*-positive cattle in the milking herd. Further screening of individual mastitic-milk samples by the University of Minnesota's Veterinary Diagnostic Laboratory[b] identified 8 cows which were actively shedding the organism. Composite milk samples were collected from each animal for 3 consecutive days and submitted for culture. Once daily during routine parlor milking in the morning, milk from the previously identified 8 cows was diverted into individual milking pails, from which 2 samples each were collected. The first sample was collected using a 30 mL sterile collection vial and refrigerated at 40 °F (4.4 °C) until submission for bacteriological evaluation. The second was collected in a 1-liter container and immediately chilled. Once chilled, the 1-liter sample was acidified with formic acid. From this 1-liter acidified sample, a 30 mL aliquot was collected and refrigerated at 40 °F (4.4 °C) for future bacteriological evaluation. All samples were refrigerated for a minimum of 48 and a maximum of 56 hours prior to laboratory processing.

Acidification of milk samples

The milk acidification process was performed in a laboratory setting. Chilled 1-liter samples of raw milk were continuously stirred on a magnetic stirrer[c] while an adjustable-volume pipette[d] was used to add 5 mL aliquots of diluted (9.8%) formic acid solution. Addition of 9.8% formic acid solution ceased when the pH meter[e] registered a value of 4.0 to 4.5. Table 1 depicts an example of the acidification process. Respective milk samples were subjected to the same acidification protocol per experimental design.

Statistical analysis

Descriptive statistics (mean, standard deviation, standard error, median, range, 25th and 75th percentiles (interquartile range, IQR), and variance) were determined for the following: culture results (cfu/mL) on 24 raw milk samples (8 cows each with 3 samples collected on consecutive days); the initial pH of each raw milk sample; the final pH of each milk sample after acidification; and the volume of dilute formic acid added to each sample in order to achieve the desired pH range. Normality of the data was assessed using the Shapiro Wilk test. Normally distributed data are presented as mean ± standard deviation. Data that were not normally distributed are presented as median and IQR. Culture results (cfu/mL) of raw and acidified milk samples were compared using the non-parametric Wilcoxon rank sum test. Significance was inferred when $P < 0.05$. All statistical analyses were performed using commercially available statistical software.[f]

Results

Sample pH and M. bovis concentration (cfu/mL)

Prior to acidification, the mean ± standard deviation pH for all milk samples obtained from the 8 cows during the 3-consecutive-day period was 6.82 ± 0.01 (range 6.60 to 7.11, n = 24). Post-acidification pH was 4.20 ± 0.03 (range 4.16 to 4.28, n = 24). The average volume of dilute 9.8%

formic acid added to a liter of milk to achieve the desired decrease in pH was 30 ± 3 mL (range 24 to 34 mL, n = 24). Prior to acidification, *M. bovis* was cultured from all 24 milk samples. Day 1 culture results for milk from the 8 cows were 100, 1,090, 1,800, 150, 680, 3,640, 15, and 155 cfu/mL of *M. bovis*, respectively. Following acidification, the samples were culture-negative for *M. bovis* ($P \leq 0.001$, Table 2). Day 2 milk culture results from the 8 cows were 20, 2,280, 550, 20, 2,110, 260, 50, and 40 cfu/mL of *M. bovis*, respectively, and like day 1, all samples were culture-negative post-acidification ($P \leq 0.001$, Table 2). Prior to acidification, culture results of day 3 milk samples from each of the 8 cows were 30, 23, 410, 40, 250, 210, 30, and 15 cfu/mL of *M. bovis*, respectively. Each sample was culture-negative for *M. bovis* post-acidification ($P \leq 0.001$, Table 2). Although the median cfu/mL in raw milk samples progressively decreased each day (day 1 median and IQR = 418 (125 to 1,445) cfu/mL, day 2 = 155 (30 to 1,330) cfu/mL, and day 3 = 35 (27 to 330) cfu/mL), overall there was no significant difference in cfu/mL between days (day 1 vs day 2, $P = 0.53$; day 1 vs day 3, $P = 0.10$; day 2 vs day 3, $P = 0.27$, Wilcoxon rank sum test).

Table 1. Acidification sequence of raw milk.

Day 1		Day 2		Day 3	
Milk volume (liter) 1.0		Milk volume (liter) 1.0		Milk volume (liter) 1.0	
Initial temperature °F (°C) 55.4° (13°)		Initial temperature °F (°C) 59.0° (15°)		Initial temperature °F (°C) 60.8° (16°)	
Initial pH 6.77		Initial pH 6.78		Initial pH 6.65	
Cumulative effect of incremental volumes of 9.8% formic acid on milk pH		Cumulative effect of incremental volumes of 9.8% formic acid on milk pH		Cumulative effect of incremental volumes of 9.8% formic acid on milk pH	
mL	**pH**	**mL**	**pH**	**mL**	**pH**
5	6.50	5	6.07	5	6.05
10	5.80	10	5.50	10	5.50
15	5.34	15	5.10	15	5.13
20	5.04	20	4.81	20	4.87
22	4.87	22	4.71	25	4.52
24	4.72	24	4.54	27	4.40
26	4.60	26	4.43	29	4.3
28	4.52	28	4.33	31	4.2
30	4.94	30	4.25		
32	4.31				
34	4.28				

Table 2. The effect of formic acid addition on the concentration of *Mycoplasma bovis* levels in milk samples collected on 3 consecutive days from cows previously diagnosed as *M. bovis* shedders. All listed values describe the observed number of colony-forming units of *M. bovis*/mL of "Raw" or "Acidified" milk.

	Day 1			Day 2			Day 3	
Cow	**Raw**	**Acidified**	**Cow**	**Raw**	**Acidified**	**Cow**	**Raw**	**Acidified**
1	100	0	1	20	0	1	30	0
2	1,090	0	2	2,280	0	2	23	0
3	1,800	0	3	550	0	3	410	0
4	150	0	4	20	0	4	40	0
5	680	0	5	2,110	0	5	250	0
6	3,640	0	6	260	0	6	210	0
7	15	0	7	50	0	7	30	0
8	155	0	9	40	0	8	15	0
Median (IQR)	418 (125-1,445)	0*	Median (IQR)	155 (30-1,330)	0*	Median (IQR)	35 (27-230)	0*

*$P < 0.001$ compared to raw milk, Wilcoxon rank sum test.

Discussion

The control of *M. bovis*-related diseases is a major management issue for the US and global cattle industry.[10] The consensus is that *M. bovis* affects all age groups of cattle through horizontal or vertical transmission, and is associated with clinical and non-clinical carrier animals.[11] Lactating cows that shed the organism in milk are significant contributors to the maintenance of *M. bovis*-related disease in dairy herds. It has been shown that feeding calves waste milk contaminated with *M. bovis* is a relevant means of disease transmission from adult cattle to youngstock.[3] Chronic asymptomatic carriers therefore contribute significantly to the maintenance of *M. bovis* within a herd.[18] In our study population of 8 cows, bacterial shedding over the 3-day-period was extremely variable from cow to cow and day to day. The highest degree of variability was observed in milk samples from cow number 6, which contained as many as 3,640 and as few as 260 cfu/mL of bacteria during a 24-hour period. In contrast, the concentration of *M. bovis* cultured from samples obtained from cow number 7 was 15, 30, and 50 cfu/mL on days 1, 2, and 3, respectively. The concentration of *M. bovis* in milk from the 8 study cows ranged from 15 to 3,640 cfu/mL throughout the 3-day study period.

The dairy industry is keen on embracing strategies geared towards preventing the propagation of *M. bovis* on farms, due largely to the lack of effective treatment for *M. bovis*-related diseases.[18] A viable preventative program may include maintaining a closed herd, screening and quarantining all purchased animals, implementing strict parlor hygiene, performing routine bulk-tank surveillance, and identifying and removing infected animals to limit disease transmission to youngstock. Strict maternity pen hygiene, early calf removal from the dam, appropriate colostrum management and feeding, proper sanitation of calf-feeding equipment, limiting nose-to-nose contact, optimal housing conditions, and adequate nutrition are all essential.

On-farm pasteurization and ultraviolet light irradiation are accepted methods proven to reduce the risk of disease transmission through waste milk.[7,14,22] Several acids (acetic, adipic, benzoic, citric, lactic, and proprionic) have been evaluated as potential milk additives.[6] The authors, however, found conflicting information regarding the use of formic acid in milk fed to calves. One source stated that its use in milk or milk replacer is currently not approved by the US Food and Drug Administration (FDA).[19] However, the FDA actually classifies formic acid as 'generally recognized as safe' (GRAS) (21 CFR 186.1316), and its use is permitted in the feed and drinking water of animals (21 CFR 573.480).[8] Proof of its efficacy in this study is therefore notable.

The current study demonstrates that the addition of dilute 9.8% formic acid to *M. bovis*-infected raw milk to a final pH of at least 4.5 effectively kills this pathogen. Calves that drink waste milk containing *M. bovis* are at risk of developing mycoplasma-associated infections and becoming carriers of the organism.[12,21] Given that *M. bovis*-related diseases are increasingly prevalent in dairies, and the practice of feeding calves waste milk is widespread, implementation of this management practice should prove useful in limiting the transmission of *M. bovis* to susceptible calf populations.

Conclusions

The objective of this case report was to evaluate whether or not formic acid could be used to control the transfer of *M. bovis* from adult cows to calves through waste milk. In addition to demonstrating the efficacy of dilute formic acid in eliminating this pathogen from fresh raw milk, results of this study also provide insight into the variable nature of *M. bovis* shedding into the mammary secretions of cattle infected with this increasingly significant agent of bovine mastitis.

Endnotes

[a]Formic Acid, Sigma-Aldrich®, Sigma-Aldrich, Inc., St. Louis, MO

[b]University of Minnesota Veterinary Diagnostic Laboratory, St. Paul, MN

[c]Flexa-Mix Magnetic Stirrer model 16, Fisher Scientific, Quebec, Canada

[d]Finnpipette™ F2 Adjustable-Volume Pipette, Thermo Fisher Scientific, Waltham, MA

[e]Accumet pH Meter model 810, Allied Fisher Scientific, Quebec, Canada

[f]Stata 14.1, Statacorp, College Station, TX

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