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Environmental Mastitis Pathogens Isolated from Bulk Tank Milk Collected on California Dairies: Prevalence and Antibiotic Susceptibility

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Abstract

The objective of this prospective study was to determine the prevalence of potential environmental bacterial mastitis pathogens in bulk tank milk, and to determine their antibiotic susceptibility patterns. Bulk tank milk samples from over 400 California dairies routinely submitting samples to the Milk Quality Laboratory were initially screened. Once potential environmental bacterial mastitis pathogens were found in bulk milk from a dairy, the dairy was repeatedly sampled on a monthly basis. Over a nine-month period, 93 dairies were identified with these bacteria, and 381 isolates were collected.

Most common isolates were *Streptococcus uberis* (42.3%), *Streptococcus dysgalactiae* (16.3%) and *Enterococcus faecium* (10.5%). Bacterial isolates (335) from 73 dairies which had at least three isolates were subjected to Minimum Inhibitory Concentration (MIC) testing using 10 antibiotics. A wide range of susceptibility to antibiotics was found. *Streptococcus dysgalactiae* tended to have lower MIC values to the test antibiotics than the other isolates. The non-streptococcal bacteria tended to have the highest MIC values.

Résumé

L'objectif de notre étude prospective était de déterminer la prévalence d'agents environnementaux pathogènes causant potentiellement la mammite dans le lait de réservoir et d'examiner leur profil de résistance aux antibiotiques. Des échantillons de lait de réservoir recueillis à partir de 400 fermes laitières de la Californie qui envoient de façon routinière des échantillons pour le contrôle laitier ont été évalués initialement. Une ferme faisait l'objet d'un échantillonnage répété à tous les mois si des agents pathogènes environnementaux causant potentiellement la mammite étaient isolés à partir du lait de réservoir. Sur une période de 9 mois, de telles bactéries étaient présentes dans 93 fermes laitières et 381 isolats ont été recueillis.

Les bactéries les plus communément isolées étaient *Streptococcus uberis* (42.3%), *Streptococcus dysgalactiae* (16.3%) et *Enterococcus faecium* (10.5%). Des isolats bactériens (335) provenant de 73 fermes laitières comportant plus de trois isolats ont été soumis à un test pour déterminer la concentration inhibitrice minimum (MIC) en présence de 10 antibiotiques. Il existait une grande variation dans le profil de résistance aux antibiotiques. La bactérie *Streptococcus dysgalactiae* montrait des valeurs de MIC plus basses que les autres en présence des antibiotiques testés. Les bactéries non streptococciques avaient les valeurs de MIC les plus élevées.

Introduction

Mastitis remains an economically significant disease of dairy cows.[2] The major portion of the economic loss is due to reduced milk production in cows with subclinical mastitis.[11] Gram-positive bacteria, such as staphylococci and streptococci, are responsible for much of this production loss. Clinical cases of mastitis due to coliform bacteria also cause economic loss. Following adoption of effective mastitis control programs and more stringent standards for milk quality, the impact of the contagious mastitis caused by gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus agalactiae* has been markedly reduced.[5,8] As contagious bacteria are more effectively controlled, environmental mastitis pathogens, such as the gram-negative coliforms and gram-positive, non-contagious streptococci, have become more important.[9] The impact of environmental bacteria has been previously reported in well-managed, low-somatic-cell-count dairy herds.[3,6,12]

In California and some other western states, environmental bacteria have emerged over the past few years to become a significant cause of both subclinical and clinical mastitis. Bacteria in this group are often reported by practicing veterinarians to be increasingly resistant to intramammary therapy, and responsible for elevated bulk tank somatic cell counts. This has been most noticeable in dairy herds with low prevalence of contagious mastitis pathogens, based on routine bulk tank culturing. In these herds, the bulk tank somatic cell counts were consistently <250,000 cells/ml, and in some cases now may exceed 350,000 cells/ml due to chronic infection with environmental bacteria.

While the negative effects of *Streptococcus uberis* and *Streptococcus dysgalactiae* have been well documented,[5,8,15] there is less information about the other streptococci, enterococci, aerococci and lactococci.[10,14] Bacteria in these genera have been found in milk samples from clinical cases of mastitis submitted for culture.[10] Most private veterinary laboratories, however, identify only *S. uberis* and perhaps *S. dysgalactiae* using standard blood agar plates,[7] and additional environmental isolates usually remain unidentified. Using blood agar methods, all other "strep-like" bacterial isolates are likely reported as environmental streptococci (E-streps) or *Streptococcus* spp, and not identified as to genus and species. The main reason for lack of additional testing to obtain a definitive identification is the time and cost of analysis required to use techniques, such as additional biochemical tests[7] or the API 20 system.[a]

The purpose of our study was to determine the prevalence of gram-positive, environmental bacteria that are potential mastitis pathogens in bulk tank milk from California dairies. In addition to specifically identifying the bacteria, we wanted to determine the antibiotic sensitivity and resistance patterns for the isolates. Our hypothesis was that the other gram-positive bacteria in the environmental group besides *S. uberis* make up a significant portion of the potential environmental bacterial mastitis pathogens, and that these other bacteria may be responsible for the lack of therapeutic response caused by antibiotic resistance.

Materials and Methods

Bacterial nomenclature – For the purposes of this study, the environmental bacteria are streptococci, other than *Streptococcus agalactiae*, enterococci, aerococci and lactococci. Gram-negative coliforms are not included. The terms "environmental strep" or "E-streps" are often used by practitioners for this group of bacteria. In this report, "environmental streptococci" will be reserved to designate only a subset of the environmental bacteria from the genus *Streptococcus*. "Non-streptococci, environmental bacteria" is used to refer to the enterococci, aerococci and lactococci.

Sampling method – Bulk tank milk samples from two large Central Valley California dairy cooperatives were cultured by the Milk Quality Laboratory (MQL) at the University of California-Davis, Veterinary Medical Teaching and Research Center in Tulare, California. Bulk tank milk samples from the cooperative dairies were routinely submitted monthly to the MQL for bacterial and mycoplasmal screening. In July 2001, milk samples from over 400 dairies were screened for environmental bacteria as they were submitted to the MQL. Dairies with samples containing at least one isolation of environmental bacteria on the initial screening were re-sampled, while those without environmental bacteria were dropped from the study. During the next nine months, an attempt was made to re-culture bulk tank milk samples from all dairies that had previously contained environmental bacteria. However, the MQL analyzes more than 150,000 milk samples per year, and this caseload made it impossible to locate each specific dairy for sampling each month.

Bacteriological culture – Presumptive identification of environmental bacteria was made using standard National Mastitis Council recommended culture procedures, including colony characteristics on blood agar, CAMP reaction, gram staining and catalase testing.[7] Environmental isolates were stored at -112°F (-80°C) for further analysis. Later, a definitive identification was made on each of the isolates using API 20, a commercially available biochemical identification system. In some cases it was not possible to identify the bacterium beyond the genus level, even with API 20.

Minimum inhibitory concentrations – Minimum Inhibitory Concentration (MIC) was performed according to the method described by National Committee for Clinical Laboratory Standards.[1] Antibiotics used for the MIC determinations were ampicillin, oxacillin, cephapirin, ceftiofur, penicillin/novobiocin, erythromycin and pirlimycin. For each antibiotic, 50 µl of bacteria was placed in dilution wells that contained 0.06, 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 or 64.0 µg/ml of the antibiotic to be tested. Each bacterial isolate was tested for each antibiotic. The MIC50 value is the mid-point or 50th percentile in the MIC distribution, while the MIC90 is the 90th percentile. A minimum of 10 isolates was required to calculate a MIC value for each antibiotic.

Results

After nine months of sampling, environmental bacteria (n=381) were identified in the bulk tank milk from 93 California dairies out of the original group of about 400 dairies. Using the API 20 technique, 13 different bacteria were identified to species, and four other

groups were only identified to genus (Table 1). On 8.6% of the dairies, only a single bacterial species was isolated, while two or three bacterial species were found on 29% of dairies; four, five or six bacterial species were found on 52.6% of dairies; and seven or eight different species were found on 9.7% of dairies. *Streptococcus uberis* and *Streptococcus dysgalactiae* were the most common isolates. Seven of the bacteria, including some only identified to genus, were found three or fewer times. Streptococci represented 68.9% of the total isolates, while enterococci represented 18%, aerococci, 6.3% and lactococci, 5.8%.

Minimum Inhibitory Concentrations for the seven antibiotics were determined for 335 (87.9%) of the 381 bacterial isolates. The MIC for some isolates was not determined because they became non-viable during storage. The non-viable cultures were *S. uberis* (14%), *S. dysgalactiae* (13%) and *E. faecium* (10%). MIC50 and MIC90 values were calculated only for bacteria with ≥ 10 isolates (Tables 2 and 3). The distribution of MIC values is shown for selected bacteria in Tables 4 and 5.

Discussion

Environmental bacteria were isolated from bulk tank milk on three or more occasions from 73 of the 93 dairies. As previously described, the focus of our study was on the environmental streptococci, enterococci, aerococci and lactococci, commonly referred to by many milk quality laboratories as "environmental streps". The distribution of environmental bacteria isolated from bulk tank milk samples in this study (Table 1) was similar to previously reported findings from bulk tank samples[14] and clinical cases of mastitis.[5,10] Therefore, it is reasonable to conclude that the environmental bacteria found in this study represented potential bacterial pathogens capable of causing mastitis. Bacteria found three or fewer times, and usually only once, on any dairy may be of little significance due to their sporadic appearance.

Table 1. Distribution of "environmental streptococci" bacterial isolates from bulk tank milk samples from California dairies.

Bacterial isolates	Number isolated (%)	Number of dairies
Aerococcus viridans	24 (6.3)	18
Enterococcus avium	3 (0.8)	2
Enterococcus durans	3 (0.8)	3
Enterococcus faecalis	10 (2.6)	9
Enterococcus faecium	40 (10.5)	29
Enterococcus spp	13 (3.4)	12
Gamella sp	1 (0.3)	1
Lactococcus lactis lactis	22 (5.8)	16
Leuconostoc spp	3 (0.8)	3
Streptococcus acidominimus	2 (0.5)	2
Streptococcus bovis	16 (4.2)	13
Streptococcus dysgalactiae	62 (16.3)	43
Streptococcus mites	2 (0.5)	2
Streptococcus salavaries	1 (0.3)	1
Streptococcus spp	9 (2.4)	7
Streptococcus suis	9 (2.4)	7
Streptococcus uberis	161 (42.3)	66

Many milk quality laboratories follow the NMC guidelines[7] for identification of streptococci in milk samples. Milk is plated on blood agar, often with CAMP-esculin. Small, smooth, translucent colonies are selected for gram stain and catalase testing. Environmental streptococci are often identified based on the colony characteristics on blood agar plates, CAMP reaction, gram staining and catalase testing. These methods presumptively identify *Streptococcus uberis*, but usually leave a significant portion of isolates labeled only as environmental streptococci. Nearly 60% of isolates in this study would have been called environmental streptococci if only standard blood agar methods were employed. These unidentified bacteria would be true streptococci not identified to species, along with the enterococci, aerococci, lactococci and others.

The finding of multiple streptococci, enterococci, aerococci or lactococci on any given dairy in our study suggests the need to utilize other testing methods beyond standard blood agar techniques to define the causative bacteria. For example, we identified 13 different bacteria using API20. *S. uberis* (42.3%) and *S. dysgalactiae* (16.3%) were the most commonly identified environmental bacteria in this study (Table 1), which was consistent with other reports.[5,9,11,14] *Enterococcus faecium* (10.5%) was frequently found in our study using API20. Streptococci species were the most frequently isolated bacteria (68.8%), while enterococci accounted for 18.1%, aerococci for 6.3% and lactococci for 5.8%. API 20 (or similar techniques) and MIC testing should be considered for use on dairies with chronically elevated bulk tank somatic cell counts and clinical mastitis cases due to environmental bacteria that are non-responsive to routine therapy.

The cost of additional diagnostic testing, such as API 20 at $25 per milk sample, should be compared to the cost of failed treatment, discarded milk, cost of antibiotics, possible missed milk quality incentives and production loss when a truly definitive diagnosis has not be made. A previous study estimated production loss of $169 ($13/cwt milk value) based on a comparison of linear scores for cows (n=1397) that were culture-positive for environmental streptococci compared to cows that were culture-negative.[13] The economic loss due to clinical mastitis has been estimated to be $100 per case.[2] Additional field research using supplemental API 20 testing in problem herds is necessary to determine how

Table 2. MIC50 and MIC90 (μg/ml) for antibiotics commonly used for intramammary infusion therapy for environmental streptococci isolated from bulk tank milk of California dairies.

Antibiotic	*S. uberis*	*S.dysgalactiae*	*S. bovis*	*Strep.* sp
Ampicillin	0.12*, 0.25**	0.06, 0.12	0.12, 0.12	0.06, 0.06
	0.06-0.5***	0.06-0.25	0.06-0.25	0.06-0.06
Oxacillin	1, 2	0.06, 0.12	0.12, 1	0.06, 0.25
	0.06-8	0.06-4	0.06-2	0.06-1
Cephapirin	0.5, 2	0.12, 0.12	0.25, 0.25	0.25 ,0.25
	0.06-64	0.06-4	0.12-0.25	0.06-2
Penicillin/novobiocin	0.12, 0.25	0.06 ,0.06	0.06, 0.12	0.06, 0.06
	0.06-1	0.06-0.12	0.06-0.12	0.06-0.06
Erythromycin	0.06, >64	0.06, 0.06	0.06, 64	0.06, 4
	0.06->64	0.06-64	0.06-64	0.06-64
Pirlimycin	0.12, 64	0.06, 2	0.06, 16	0.06, 64
	0.06-64	0.06-64	0.06-16	0.06-64
Number of isolates	139	55	16	16

[a] MIC50* is followed by MIC90**.
[b] The ranges from lowest to highest MIC*** value are shown below the MIC50 and MIC90 values.

Table 3. MIC50 and MIC90 (μg/ml) for antibiotics commonly used for intramammary infusion therapy for non-streptococci, environmental bacteria isolated from bulk tank milk of California dairies.

Antibiotic	*E. faecium*	Lactococci	Aerococci	*Entero.* sp
Ampicillin	0.5*, 1**	0.12, 0.5	0.06, 0.12	0.5, 1
	0.06-2***	0.06-0.5	0.06-0.25	0.06-1
Oxacillin	4, 64	1, 4	4, 16	8, 16
	0.12-64	0.25-8	0.5-64	8-64
Cephapirin	32, 64	4, 4	0.5, 8	8, 64
	0.25-64	1-8	0.25-8	0.06-64
Penicillin/novobiocin	0.25, 1	0.12, 0.5	0.12, 0.25	0.25, 1
	0.06-1	0.06-0.5	0.06-0.25	0.06-1
Erythromycin	1, 8	0.06, 64	0.12, 64	0.5, 2
	0.06->64	0.06-64	0.06-64	0.06-2
Pirlimycin	4, 8	0.12, 64	0.06, 0.25	0.06, 32
	0.06-64	0.06-64	0.06-0.25	0.06-32
Number of isolates	36	21	20	12

[a] MIC50* is followed by MIC90**.
[b] The ranges from lowest to highest MIC value*** are shown below the MIC50 and MIC90 values.

an altered treatment regime might effect the economic outcome compared to no additional API 20 testing.

The need for antibiotic sensitivity and resistance testing is clearly shown by the diversity of environmental isolates and the range of MIC values for different antibiotics. For example, the MIC90 for cephapirin, the most commonly used intramammary antibiotic used to treat mild mastitis in dairies in our study, was 0.12 μg/ml for *S. dysgalactiae*, 2.0 μg/ml for *S. uberis*, 4.0 μg/ml for lactococcus, 8 μg/ml for aerococcus and 64 μg/ml for *E. faecium* (Tables 2 and 3). Much higher MIC values for cephapirin were found for the non-streptococci, environmental bacteria (Table 3) compared to the streptococci (Table 2). Of the environmental streptococci, *S. dysgalactiae* was the most sensitive to the commonly used intramammary infusion antibiotics, and the other streps were similar in susceptibility to these antibiotics. There was variation within the MIC90 values for the antibiotics among the non-streptococci, environmental bacteria, and these MIC90 values were generally

Table 4. Distribution of MIC values (μg/ml) by dilutions[a] for *Strep. uberis* and *Strep. dysgalactiae* for commonly used intramammary antibiotics for mastitis.

Bacteria	Antibiotic[b]	0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>64
Strep. uberis	Amp	28	**53**	54	4	0	0	0	0	0	0	0	0
	Oxa	4	7	7	11	**60**	43	3	4	0	0	0	0
	Cep	2	10	25	**52**	42	2	1	2	1	0	1	0
	P/nov	52	**66**	17	3	1	0	0	0	0	0	0	0
	Eryth	**80**	3	2	2	2	8	8	2	1	0	0	30
	Pir	62	**11**	2	0	6	13	13	1	4	3	13	11
Strep. dysgalact	Amp	49	5	1	0	0	0	0	0	0	0	0	0
	Oxa	**45**	5	2	0	2	0	1	0	0	0	0	0
	Cep	4	**46**	3	0	1	0	1	0	0	0	0	0
	P/nov	**51**	3	0	0	0	0	0	0	0	0	0	0
	Eryth	**50**	1	2	1	0	0	0	0	0	0	0	1
	Pir	**43**	5	1	0	0	2	2	1	0	0	0	1

[a] **Bold** numbers are MIC50 dilutions and underlined numbers are MIC90 dilutions.
[b] Amp = ampicillin; Oxa = oxacillin; Cep = cephapirin; P/nov = penicillin/novobiocin; Eryth = erythromycin; and Pir = pirilimycin.

Table 5. Distribution of MIC values (μg/ml) by dilutions[a] for *Entero. faecalis* and *Entero. faecium* for commonly used intramammary antibiotics for mastitis.

Bacteria	Antibiotic[b]	0.06	0.12	0.25	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	>64
Entero. *faecalis*	Amp	0	0	2	**5**	3	0	0	0	0	0	0	0
	Oxa	0	0	0	0	0	1	1	3	**6**	0	0	0
	Cep	0	0	0	0	0	0	1	3	**2**	4	0	0
	P/nov	0	1	1	2	**6**	0	0	0	0	0	0	0
	Eryth	3	0	0	**5**	1	1	0	0	0	0	0	0
	Pir	1	0	1	0	**3**	1	1	0	1	1	0	0
Entero. faecium	Amp	1	5	3	**14**	9	4	0	0	0	0	0	0
	Oxa	0	1	1	2	2	4	**8**	9	3	1	5	0
	Cep	0	0	1	1	0	2	4	2	3	**11**	12	0
	P/nov	2	6	**20**	6	2	0	0	0	0	0	0	
	Eryth	8	5	0	3	**6**	8	2	2	0	0	0	0
	Pir	5	10	0	1	**4**	2	3	7	1	1	2	0

[a] **Bold** numbers are MIC50 dilutions and underlined numbers are MIC90 dilutions.
[b] Amp = ampicillin; Oxa = oxacillin; Cep = cephapirin; P/nov = penicillin/novobiocin; Eryth = erythromycin; and Pir = pirilimycin.

higher than those for the environmental streptococci. Several bacteria had MIC values across the entire range of MIC test dilutions (Table 4 and 5). This may indicate the need for species identification along with antibiotic susceptibility testing in herds with significant mastitis problems due to environmental streptococci. The MIC values for ceftiofur (data not shown), which may be used in an extra-label manner for the treatment of mastitis, were similar to those for cephapirin for the streptococci (Table 2). However, the MIC values for ceftiofur were higher than most antibiotics tested in this study (Table3) for the non-streptococci, environmental bacteria.

Interestingly the enterococcal isolates included *Enterococcus faecalis* and *Enterococcus faecium*. These bacteria are known for their ability to acquire and transfer elements that confer resistance to antibiotics.[4] These enterococci could potentially serve as reservoirs for antibiotic resistant genes for other bacteria residing in similar environmental niches on dairies. The antibiotic susceptibility patterns of non-streptococcal isolates in this study suggests they could be the cause of antibiotic treatment failures being reported by practicing veterinarians dealing with mastitis caused by environmental streptococci.

While not routinely necessary to spend additional money for antibiotic susceptibility testing, it may be prudent to more precisely define the therapeutic regime when environmental streptococci are causing herd mastitis problems. In an unpublished survey of 49 of the study dairies with both multiple bacterial isolates and

MIC testing, we found that 79% of the dairies were using a single intramammary infusion antibiotic for mild cases of mastitis, which was usually cephapirin or pirlimycin. Our findings showed that on nearly 80% of the study dairies, three or more different environmental streptococci were found in the bulk tank milk samples. The distribution of MIC values within the various dilutions of antibiotics may also create situations where a disproportionate number of bacterial isolates may have high MIC90 values (Tables 3 and 4).

The direct usefulness of MIC50 and MIC90 values is limited by several factors. First, the relationship between the dose of antibiotic administered and the concentration achieved within the mammary gland for a given antibiotic infusion product is not commonly known to the practitioner or dairy producer. Under typical circumstances, the user of the product must rely on the manufacturer to provide information on antibiotic dosage to achieve the appropriate level at the site of infection. A second limitation is the scarce amount of information on MIC values for environmental streptococci from actual cases of mastitis in dairy cows. Furthermore, the majority of break-points used to determine susceptibility or resistance are based on human data. For these reasons, use of MIC values may be limited to detecting relative changes in susceptibility for assisting the practitioner in making empirical selections of antibiotics.

Conclusions

Repeated finding of multiple gram-positive, environmental bacterial mastitis pathogens with a wide diversity of MIC values demonstrated the need to define the causative bacteria, and their probable antibiotic susceptibility pattern, in order to optimize the antibiotic therapy program for herds that have mastitis problems caused by environmental streptococci. This may be particularly prudent when gram-positive, environmental bacteria other than *S. uberis* and *S. dysgalactiae* are present.

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Footnote

[a] BioMeriuex-Vitek Inc, Hazelwood, MO

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