Northern NY Agricultural Development Program
2013-14 Project Report

Identification, Distribution, and Characterization of Mastitis-Causing Pathogens Previously Identified as "Other *Streptococcal* Species"

Project Leader(s):

Jessica C. Scillieri Smith, DVM
Quality Milk Production Services
Northern New York Regional Laboratory
Animal Health Diagnostic Center
Cornell University

34 Cornell Drive
Canton, NY 13617
(315)379-3930
Jcs385@cornell.edu

Collaborator(s):

Paolo Moroni, DVM, PhD
Carlos Santisteban, DVM
Brad Rauch, MS
Daryl Nydam, DVM, PhD
Quality Milk Production Services
Animal Health Diagnostic Center
Cornell University

Mark Thomas, DVM
Countryside Veterinary Clinic
Dairy Health Management Services
Lowville, NY
mthomas@dairyhealthmanagement.com

Kimberly Morrill, PhD
Cornell Cooperative Extension
Northern NY Dairy Specialist
kmm434@cornell.edu

John Kingston and Jordan Fisher
Mapleview Dairy, LLC
Madrid, NY

Cooperating Producers:

County	Number of Farms
Clinton	4
Essex	3
Franklin	9
Jefferson	15
Lewis	21
St. Lawrence	29
Total	**81**

Background:

Environmental organisms cause the majority of clinical mastitis infections (Hogan, 1989) in herds with low somatic cell count (SCC), including 25.4% of which are environmental *Streptococci,* specifically *Streptococcus* species other than *Streptococcus agalactiae.*

Because these organisms are predominantly associated with environmental sources for infection and risk of infection is not isolated to milking, even cows on very well managed dairy farms are at risk for these infections.

Gram-positive, catalase-negative cocci account for a large number of cases of clinical and subclinical mastitis in the dairy industry (Jones, 2009). *Streptococcus uberis* is the most commonly isolated pathogen from cows with clinical mastitis in Flanders at 18% of samples, *Streptococcus dysgalactiae* was identified in 7.2% of samples and other esculin-positive cocci other than *Strep uberis* were identified in 2.1% of samples (Verbeke, 2014).

When evaluating mastitis on five farms in New York State, the largest percentage of clinical mastitis cases were attributed to *Streptococcus* species, regardless of which case of mastitis the cow was experiencing during the lactation (Hertl, 2014).

These environmental Gram-positive, catalase negative organisms can be associated with chronic mastitis late in lactation (Wyder, 2011), especially *Lactococcus garviaeae*, with increases in SCC when compared to control animals, indicating that this organism may need to be considered a mastitis causing pathogen.

Lacotoccus lactis appears to be difficult to differentiate phenotypically from other *Streptococcus* organisms, indicating that this group is likely misidentified and unreported as a potential cause for clinical mastitis (Plumed-Ferrer, 2013; Werner, 2014).

Five farms (in New York and Minnesota) with high levels of chronic mastitis initially identified as *Streptococcus* species were identified using PCR DNA amplification, showed that 70% of samples were *Lactococcus lactis* ssp. *Lactis*, 17% were *Enterococcus saccharolyticus,* and the remaining 13% of samples were *Streptococcus uberis* (7%), *Enterococcus faecium* (1.5%), *Enterococcus* ssp (1.5%), *Lactococcus lactis* (1.5%) and *Streptococcus dysgalactiae* (1.5%) (Werner, 2014).

There is the potential that *Lactococcus* and other organisms identified as "other" *Streptococcus* species are significant pathogens on dairy farms and their pathogenicity may be different than other organisms within this category. If farm management would be different for these pathogens, positive identification for the dairy farm will help direct treatment or culling decisions, resulting in a potential reduction in antibiotic use, waste milk and chronically infected cows.

Identification of *Streptococcus agalactiae* and *Strep dysgalactiae* using standard microbiological methods are very accurate when compared to 16S sequencing (Wyder, 2011). However, this group of organisms contains a large number of other *Streptococcus*

organisms, in addition to including the genus *Lactococcus¸ Enterococcus*, and *Aerococcus*, which cannot be easily or economically differentiated using biochemical tests (Fortin, 2003).

While identification of *Streptococcus dysgalactiae* using biochemical tests agrees with diagnosis via the API 20 STREP strip test, over 90% of the time, identification of *Streptotoccus uberis, Aerococcus* species, *Enterococcus* species and *Lactococcus* species are only correctly identified 23- 70% of the time with biochemical tests. As a result, these pathogens are frequently grouped together as "*Streptococcus* species," making it difficult to assess the clinical significance of these organisms as mastitis causing pathogens (Devriese, 1999).

In addition, it appears that identification of *Streptococcus uberis* can be difficult using only biochemical tests (Odierno, 2006), requiring 11 different reagents in order to get a 94% accuracy rate, which can be cost prohibitive for many diagnostic laboratories. Of those identified as *Streptococcus uberis*, 24% were identified as *Lactococcus* spp., 5% as *Enterococcus* spp., and 2% as *Aerococcus* spp (Fortin, 2003). In an analysis of sheep bulk tank milk, all gram-positive, catalase-negative cocci were identified to the genus and species level. A total of 23 species were identified using 16S rDNA sequencing, belonging to five genera including *Enterococcus, Streptococcus, Lactococcus, Aerococcus* and *Trichococcus* (de Garnica, 2014).

Methods:

Milk samples submitted to Quality Milk Production Services **(**QMPS; Canton, NY), Countryside Veterinary Clinic (Lowville, NY), or cultured using the Minnesota tri-plate on-farm culture system (Mapleview Dairy, Murcrest Dairy, and Hilltop Dairy) were cultured using standard microbiological methods established by the National Mastitis Council (NMC Handbook, 1999). For a six-month period (May to November, 2014), those samples which were diagnosed as *Streptococcus dysgalactiae*, *Streptococcus uberis* or *Streptococcus* species were sent overnight to Cornell University Animal Health Diagnostic Center in Ithaca, NY.

All confirmed samples were then speciated using Matrix Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF, Bruker Daltonics, The Woodlands, TX) technology to determine the bacteria present to the genus and species level. Bacteria for which the MALDI-TOF results have not been verified or for which the MALDI-TOF could not reliably confirm identity of were then identified by the QMPS Molecular Lab using Rapid PCR technology.

All samples included in the study were tested for somatic cell count using a DeLaval Cell Counter (DCC, DeLaval Inc., Tumba, Sweden). Results were either numerical or resulted in a "Flow Error." Numerical results were transformed into Linear Score (**LS**), using the equation $\log_2(SCC/100{,}000) + 3$.

All cows with samples submitted by farms with monthly Dairy Herd Improvement Association (DHIA) testing were tracked in Dairy Comp 305 to monitor SCC for the

following two test days, days in milk (DIM) at the time of sampling and if treatment was administered (including product and number of days treated if known). Isolates from cows with infections caused by organisms other than Strep dysgalactiae and Strep uberis were run for Mean Inhibitory Concentration (MIC) to determine antibiotic sensitivity profiles.

Results:

A total of 471 milk samples cultured positive for Gram-positive, catalase-negative, non-β hemolytic cocci (GPCNC) and speciated to the genus and species level. Samples were submitted by Countryside Veterinary Clinic (n=19), collaborating farms (n=58) and QMPS Canton (n=444). During that time, QMPS Canton processed 8,361 samples for aerobic culture from 143 dairy farms. Of the samples that cultured positive for Gram-positive, catalase-negative, non-β hemolytic cocci, 155 were identified as *Streptococcus dysglactiae* (33%), 150 as *Streptococcus uberis* (32%), 112 as *Lactococcus lactis* (24%), 16 as *Lactococcus garviae* (3.4%), 22 as *Enterococcus saccharolyticus* (4.7) and 16 as other *Streptococcus*, *Enterococcus* and *Aerococcus* species (Table 1).

Samples originated from whole herd surveys performed by QMPS (n =106; 82 from voluntary surveys (21%) and 24 (5%) from required surveys), submitted clinical mastitis samples (n=292), submitted from fresh cows (n =10), submitted subclinical mastitis (n=15) and sampled for unknown reasons (n=72; Figure 1).

Initial Linear Score

All milk samples were tested for SCC using the DCC after the culture was identified as *Streptococcus* (non-*agalactiae*) infection. Results from only quarter samples were compared in order to remove the effect of milk from potentially healthy quarters in composite samples. Quarter samples (n=244) were tested with the DCC, of which 144 yielded a numerical result and 100 yielded a result of "Flow Error" (FE). Over half of *Streptococcus uberis* (52%, n=42) and *Streptococcus dysgalactiae* (54%, n=100) yielded a result of FE. A similar percentage (54%) of *Enterococcus* species resulted in FE (n=13). However, a much lower percentage of *Lactococcus* yielded this result (15% of 80 samples for *L. lactis* and 0% of 4 samples for *L. garviae*). Out of the remaining samples which yielded a numerical result, the average DCC for all organisms was over 1 million cells/ml, with an average LS ranging from 6.7 (*Enterococcus*) to 7.68 (*Lactoccus garviae*; Figures 2 and 3).

Days in Milk at Sampling

Days in milk at the time of sampling for all cows from farms with DHIA records was recorded. The average DIM from cows with clinical mastitis was used to calculate the average DIM because other samples were potentially taken at arbitrary time points which do not correlate with the presence of the infection. Average DIM was only calculated for organisms with ten or more cows with clinical mastitis (quarter) samples. *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Lactococcus lactis* all showed a range of time points for infection (early to late lactation). *Enterococcus saccharlyticus* was only found in mid and late lactation animals from clinical quarters; however, it was identified in early lactation animals where composite samples were submitted. The average DIM for

organisms was during the mid-lactation period - *Streptococcus dysgalactiae* (162 DIM), *Streptococcus uberis* (96 DIM), *Lactococcus lactis* (170 DIM), and *Enterococcus saccharlyticus* (140 DIM; Figure 4).

Somatic Cell Count Resolution

Somatic cells count from DHIA records was tracked for cows from herds that participate in monthly DHIA testing. In addition, treatment information from cows with clinical mastitis was gathered from farms that recorded treatment product and duration in Dairy Comp 305. Cows were assessed for SCC resolution by evaluating their SCC on Dairy Comp 305 if they had a test day between 15 and 45 d after sampling and there was more than five d between the end of treatment and the test day. Somatic cell count resolution was defined as a SCC < 200,000 cells/ml. Treatment product or duration of therapy was not taken into account for this analysis.

There was a significant range in percent of animals with SCC resolution when comparing infections caused by the different organisms. *Streptococcus dysglactiae* and *Streptococcus uberis* both showed a higher SCC resolution (70% of 80 cows and 77% of 13 cows respectively). *Lactococcus lactis* showed a lower rate of resolution with only 33% of animals showing a SCC resolution (57 cows). *Lactococcus garviae* had a 100% SCC resolution rate, but there were only two cows that met the criteria for evaluation. *Enterococcus* species were grouped because of the low sample size (n=10) but only showed a 30% resolution rate. When comparing *Streptococcus dysgalactiae* and *Lactococcus lactis*, there was a significant difference in the response to therapy ($p<0.001$; Figure 5).

Risk of Leaving the Herd

Cows that left the herd prior to the second test day after sampling were recorded. This parameter was used to evaluate the 336 cows from 42 farms with monthly DHIA testing. Of the 198 cows with *Streptococcus* infections (*Streptococcus dysgalactiae, Streptococcus uberis* other *Streptococcus* species), 20 cows were no longer in the herd by the second test day (10%). Twenty two cows from the 104 cows with *Lactococcus lactis* and *Lactococcus garviae* left the herd by the second test day (21%). The difference between the risk of leaving the herd after a *Streptococcus* infection when compared to a *Lactococcus* infection was significant ($p<0.01$;Figure 6).

Mean Inhibitory Concentrations

Mean Inhibitory Concentration results can be seen in Figures 7-9 for *Lactococcus lactis*, *Lactococcus garviae* and *Enterococcus saccharlyticus.* Some strains of *Lactococcis lactis* (12 out of 42) and *Lactococcus garviae* (5 out of 12) appear to be resistant to tetracycline, and many *Lactococcus garviae* strains (10 out of 12) exhibit a resistant pattern to Pirlimycin. One strain of *Enterococcus saccharolyticus* was resistant to Pirlimycin (out of 19 tested).

Conclusions/Outcomes/Impacts:

Lactococcus appears to be a potentially important mastitis causing pathogen, identified in over 23% of non-β hemolytic, catalase negative, Gram-positive cocci infections

diagnosed by QMPS Canton and Countryside Veterinary Clinic between May and November, 2014 on 19 farms in the region. It appears more likely to result in a chronically infected cow and increase the risk of that cow leaving the milking herd by the second test day after sampling.

Lactococcus is not currently identified using standard microbiology techniques and is identified as "other" *Streptococcus* species. Additional information is necessary to further characterize this infection so that more specific and helpful management recommendations can be made when this infection is identified.

In addition, QMPS is working on developing more specific and sensitive microbiology testing in order to accurately identify these infections for the producer.

Outreach:

Results have been presented at the following meetings:

- 11th annual Northern New York Dairy Institute - January 13-15, 2015, Lowville, Canton and Chazy, NY
    - 50 participants representing 27 farms with over 12,000 dairy cows
- NNYADP meeting - January 30, 2015, Watertown, NY
- Northeast Dairy Medicine Symposium – March 22, 2015, Syracuse, NY
- National Mastitis Council Annual Meeting – January, 2015

Results will also be presented at:

- Vermont Veterinary Medical Association meeting – June, 2015, Burlington, VT
- National Mastitis Council Regional Meeting – July, 2015, Syracuse, NY
- American Association of Bovine Practitioners, Milk Quality preconference seminar – September, 2015, New Orleans, LA

Next Steps:

Many of the species identified in this study have not been considered important pathogens in bovine mastitis and the role of these microorganisms as economically important agents remains unclear. Because we have been able to more accurately identify these pathogens using MALDI-TOF technology, we have determined that for some farms, there are larger numbers of animals infected with these lesser known pathogens. When looking at five farms with the highest percentage of cases of mastitis caused by non-β hemolytic, catalase negative, Gram-positive cocci species (previously all categorized as "*Streptococcus* species") in the study done in 2014, each farm appears to have a different distribution of mastitis-causing organisms. For some farms, *Lactococcus* represents over 50% of cases of mastitis caused by these organisms.

A follow up study characterizing the outcome of cases of clinical and subclinical mastitis caused by these organisms would add useful information which will help dairy farms make management decisions after diagnosis. Further knowledge is needed on bacteriological and SCC response to therapy and risk of culling will be helpful for farms making management decisions for these cows, especially if these infections are common in a herd. These results will lead to rational, science-based assessment of the clinical and economic importance of these organisms and their direct impact on NNY dairies.

Acknowledgments:

This project would not have been possible without the assistance of the dairy farms in Northern NY who submitted samples or on farm culture plates to QMPS and allowed us to resample their cows for follow up microbiology. A special thanks to the staff of Quality Milk Production Services in Canton, NY along with our summer intern, Chelsea Metcalf. In addition, we appreciate the support from Countryside Veterinary Clinic, allowing us to include their culture samples into this project.

Reports and/or articles:

- A presentation on the results of the study was presented during the 11th Northern New York Dairy Institute – Reproduction. All producers in attendance received a copy of the slides.
- "Emerging Pathogens: The Latest Information on Klebsiella, Prototheca and Lactococci." National Mastitis Council Annual Meeting Proceedings, 2015.
- "QMPS Research Update: An investigation into *Streptococcus* species mastitis in Northern New York." Northeast Dairy Production Medicine Symposium Proceedings, 2015.
- Planned publication in Hoards Dairyman in July, 2015
- Planned peer-reviewed manuscript to Journal of Dairy Science

Person(s) to contact for more information:

Jessica C. Scillieri Smith, DVM
Quality Milk Production Services
34 Cornell Drive
Canton, NY 13617
(315)379-3930
Jcs385@cornell.edu

Table 1: Distribution of organisms tested.

Organism	#Farms	#Cases	% of cases
Strep dysgalactiae	35	155	32.91%
Strep uberis	76	150	31.85%
Lactococcus lactis	19	112	23.78%
Lactoccus garvieae	11	16	3.40%
Streptococcus parauberis	3	3	0.64%
Streptococcus suis	1	1	0.21%
Streptococcus gallolyticus	4	4	0.85%
Streptococcus acidominimus	1	1	0.21%
Streptococcus oralis	1	1	0.21%
Enterococcus saccharolyticus	13	22	4.67%
Enterococcus faecium	1	1	0.21%
Enterococcus casseliflaves	1	1	0.21%
Enterococcus facecalis	3	3	0.64%
Enterococcus cecorum	1	1	0.21%
Aerococcus viridans	2	2	0.42%
Unspeciated Strep	5	7	1.49%
Total		**471**	

Table 2. Mean inhibitory concentrations of the nine antimicrobials tested on 42 *Lactococcus garvieae* isolates. Resistance breakpoints are highlighted in black and intermediate in grey. Antibiotics where no black or grey is indicated have no breakpoint in the literature. Reference for the breakpoint used are indicated by the letter in row two and listed at the end of the table.

		No. of strains with MIC (µg/mL)									
Antimicrobials	Breakpoint Reference[1]	0.12	0.25	0.50	1	2	4	8	16	32	64
Penicillin	a	2		1	9						
Ampicillin	a	1	1	3	6	1					
Oxacillin+2%NaCl	b					6	1	5			
Cephalothin	c					2	3	7			
Ceftiofur	d			12							
Erythromycin	a		11	1							
Penicillin/Novobiocin	d				12						
Pirlimycin	d			1	1		3	7			
Tetracycline	e				7			5			

[1]a) CLSI resistance breakpoint for enterococci - b) no breakpoints available - c) CLSI resistance breakpoint for all microorganisms - d) CLSI resistance breakpoint for cattle mastitis - e) EUCAST breakpoints for *Lactococcus lactis*

Table 3. Mean inhibitory concentrations of the 9 antimicrobials tested on 42 *Lactococcus lactis* isolates. Resistance breakpoints are highlighted in black and intermediate in grey. Antibiotics where no black or grey is indicated have no breakpoint in the literature. Reference for the breakpoint used are indicated by the letter in row 2 and listed at the end of the table.

		No. of strains with MIC (µg/mL)									
Antimicrobials	Breakpoint Reference[1]	0.12	0.25	0.50	1	2	4	8	16	32	64
Penicillin	a	5	27	7	3					5	
Ampicillin	a	23	15	3	1					23	
Oxacillin+2%NaCl	b					42					
Cephalothin	c					40	1	1			
Ceftiofur	d			41		1					
Erythromycin	a		42								
Penicillin/Novobiocin	d				41	1					
Pirlimycin	d			42							
Tetracycline	e				30				12		

[1]a) CLSI resistance breakpoint for enterococci - b) no breakpoints available - c) CLSI resistance breakpoint for all microorganisms - d) CLSI resistance breakpoint for cattle mastitis - e) EUCAST breakpoints for *Lactococcus lactis*

Table 4. Mean inhibitory concentrations of the 9 antimicrobials tested on 19 *Enterococcus saccharolyticus* isolates. Resistance breakpoints are highlighted in black and intermediate in grey. Antibiotics where no black or grey is indicated have no breakpoint in the literature. Reference for the breakpoint used are indicated by the letter in row 2 and listed at the end of the table.

		No. of strains with MIC (µg/mL)									
Antimicrobials	Breakpoint Reference[1]	0.12	0.25	0.50	1	2	4	8	16	32	64
Penicillin	a	7	2	2	7	1					
Ampicillin	a	8		3	8						
Oxacillin+2%NaCl	b					8	1	10			
Cephalothin	c					7	7	5			
Ceftiofur	d			13	3	3					
Erythromycin	a		19								
Penicillin/Novobiocin	d				19						
Pirlimycin	d			14	2	2	1				
Tetracycline	a				7	1		11			

[1]a) CLSI resistance breakpoint for enterococci - b) no breakpoints available - c) CLSI resistance breakpoint for all microorganisms - d) CLSI resistance breakpoint for cattle mastitis - e) EUCAST breakpoints for *Lactococcus lactis*

Figure 1 -Sample types

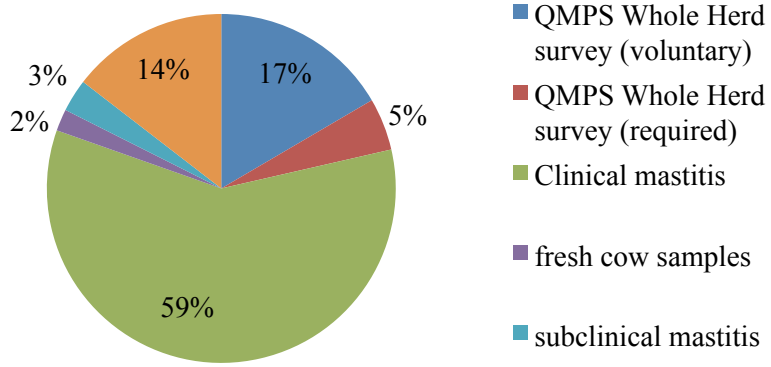
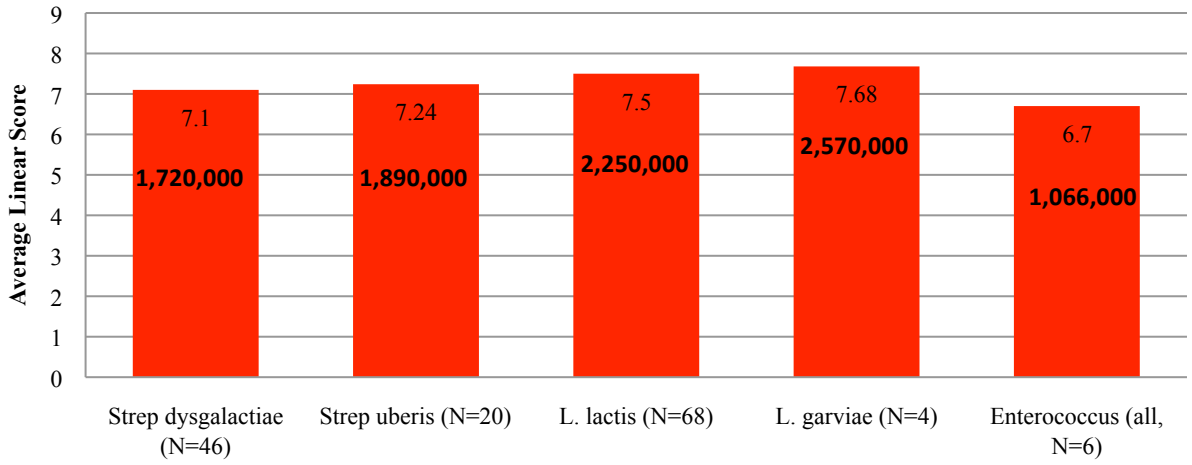


Figure 2- Average Initial Linear Score by Organism



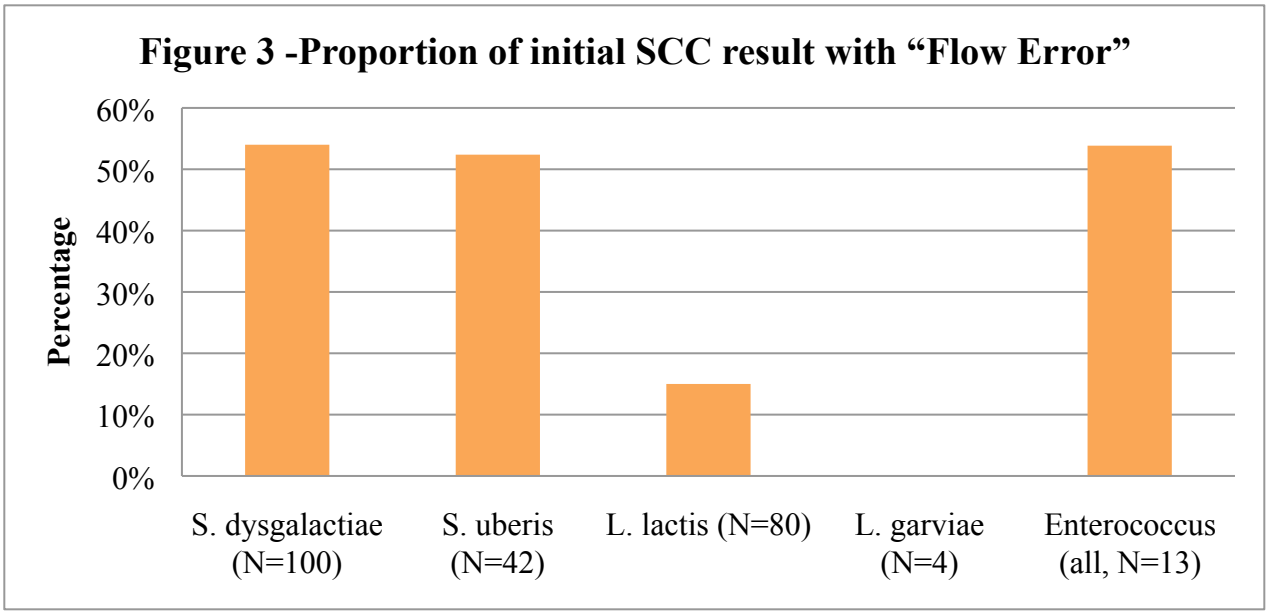


Figure 3 -Proportion of initial SCC result with "Flow Error"
Percentage
60%
50%
40%
30%
20%
10%
0%
S. dysgalactiae (N=100)
S. uberis (N=42)
L. lactis (N=80)
L. garviae (N=4)
Enterococcus (all, N=13)

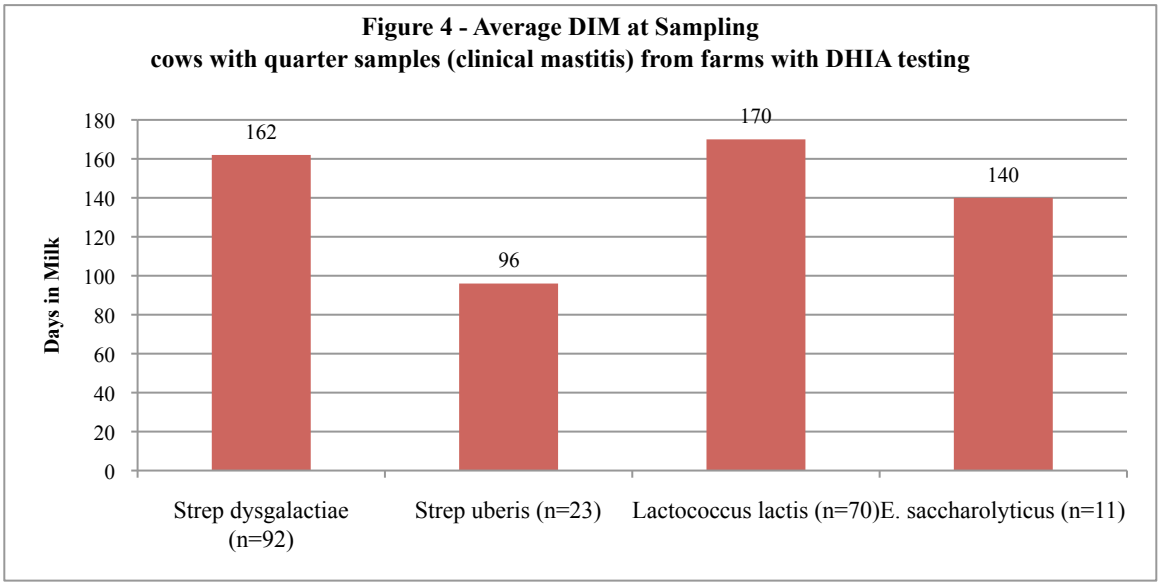
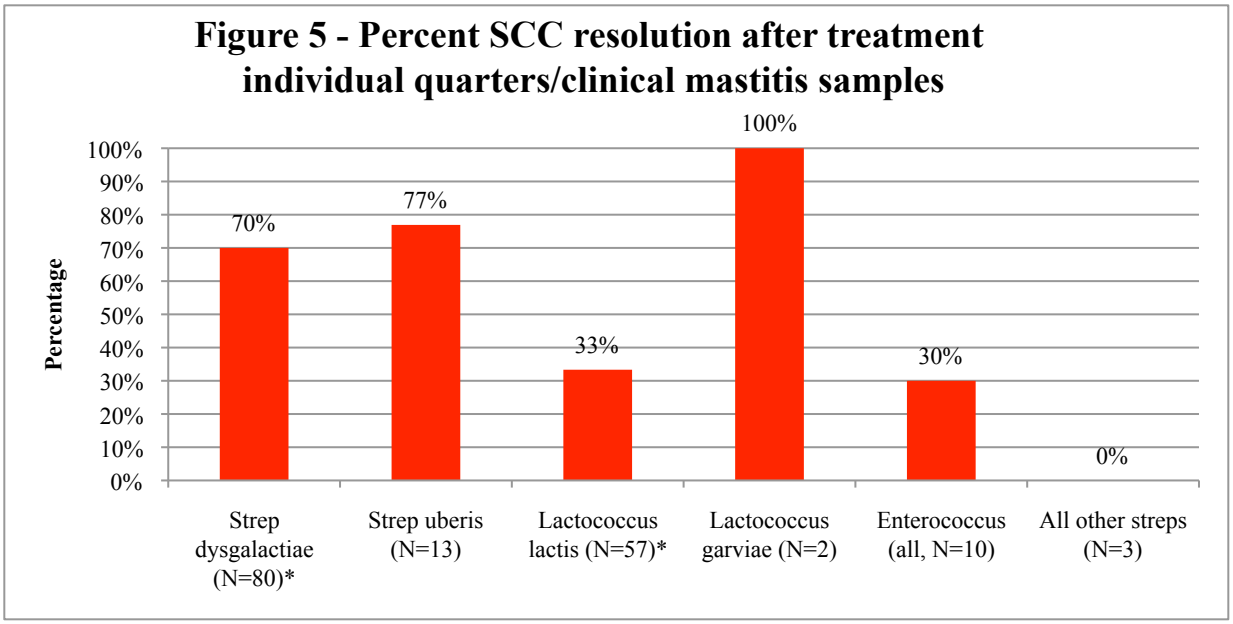
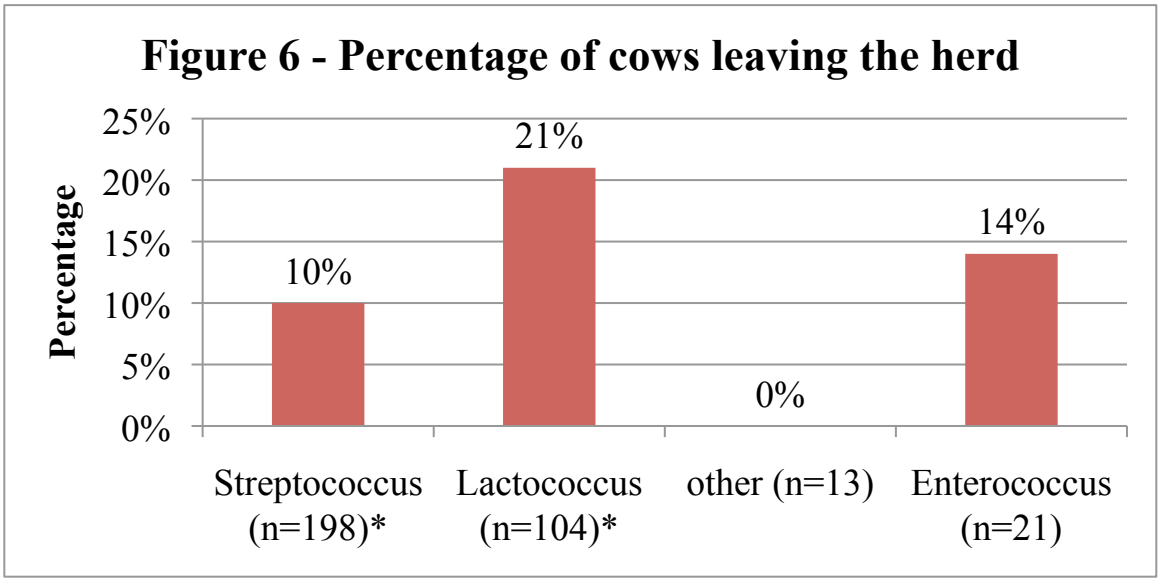


Figure 4 - Average DIM at Sampling
cows with quarter samples (clinical mastitis) from farms with DHIA testing
Days in Milk
180
160
140
120
100
80
60
40
20
0
162
96
170
140
Strep dysgalactiae (n=92)
Strep uberis (n=23)
Lactococcus lactis (n=70)
E. saccharolyticus (n=11)





Bibliography

de Garnica, M. L.-N. (2014). Diversity of gram-positive catalase-negative cocci in sheep bulk tank milk by comparative 16S rDNA sequence analysis. *International Dairy Hournal*, 142-145.

Devriese, L. J. (1999). Identification of aesculin-hydrolyzing streptococci, lactococci, aerococci and enterococci from subclinical intramammary infections in dairy cows. *Veterinary Microbiology*, 87-90.

Fortin, M. S. (2003). Identification of Catalase-Negative, Non-Beta-Hemolytic, Gram-Positive Cocci isolated from milk samples. *Journal of Clinical Microbiology*, 106-109.

Hertl, J. A. (2014). Pathogen-specific effects on milk yield in repeated clinical mastitis episodes in Holstein dairy cows. *Journal of Dairy Science*, 1465-1480.

Hogan, J. S. (1989). Field Survey of Clinical Mastitis in Low SOmatic Cell Count Herds. *Journal of Dairy Science.*, 1547-1556.

Jones, G. M. (2009). *Virginia Cooperative Extension publication number 404-234.* Retrieved January 5, 2015, from Virginia Cooperative Extension: http://pubs.ext.vt.edu/category/dairy-cattle.html

Odierno, L. L. (2006). Conventional identification of Streptococcus uberis isolated fomr bovine mastitis in Argentinean dairy herds. *Journal of Dairy Science*, 3886-3890.

Plumed-Ferrer, C. K. (2013). Characterization of Lactococcus lactis isolated form bovine mastitis. *Veterinary Microbiology*, 592-599.

Verbeke, J. S. (2014). Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. *Journal of Dairy Science*, 6926-6934.

Werner, B. P.-A. (2014). Short communication: Genotypic and phenotypic identification of environmental streptococci and association of Lactococcus lactis ssp. lactis with intramammary infections among different dairy farms. *Journal of Dairy Science*, ??

Wyder, A. B. (2011). Streptococcus spp. and related bacteria: Their identification and their pathogenic potential for chronic mastitis - A molecular approach. *Research in Veterinary Science*, 349-357.