Notes for 10-herd NAS manuscript:

**Methods**

Would I be able to do a BLCM-ish analysis incorporating Se and Sp values for culture, to help correct for some misclassification bias?

Interpretation of duplicate quarter-milk samples in series results in decreased sensitivity but higher specificity for identifying non-*aureus* staphylococci intramammary infections as compared to a single sample (Dohoo et al., 2011). This approach was chosen to maximize the specificity of culture to identify quarters as positive for a non-*aureus* staphylococci IMI (i.e., minimize false positives).

Collection of a large number of samples in the field under time pressure and occasionally with minimally trained personnel resulted in a moderately high rate of contamination (13%).

A readily-available, reliable bench-top test has not yet been developed for differentiating NASM species, even though it is established that some species are more relevant to udder health than others. While the technology exists for accurate speciation of NASM at larger diagnostic labs and research settings (MALDI-TOF, PCR), currently, most NASM species are only able to be lumped together as “non-*aureus* staphylococci” when cultured in labs without the resources or infrastructure to speciate isolates (e.g., on-farm culture, veterinary practices). However, it is still unclear whether treatment is warranted for these infecitons at all.

Future work towards developing more readily available methods of speciation may better inform treatment decisions for producers, allowing them to treat or cull animals with infections due to more problematic SaM and withhold treatment for those of less concern. This may be particularly important if future research resolves the current lack of understanding of the relationship between NASM IMI, risk of infection, elevation of SCC, and effect on milk yield. For example, Chen et al. reported lower daily milk losses among first parity cattle with moderate SCC increases (200,000 cells/mL) compared to greater losses among older cows with SCC greater than 500,000 cells/mL.

For example, Chen et al. reported milk yield losses for first lactation animals increased from 0.42 kg/day to 0.97 kg/day as SCC increased from 200,000 cells/mL to 500,000 cells/mL; 500-800 was 0.97 kg/day to 1.26. this disproportionate increase in milk loss as SCC increases was even more pronounced in later lactation cows. The relationship between IMI, elevated SCC, and milk yield is complex. It is uncontroversial that as SCC increases, milk loss increases (chen, shook); however, this is offset with the fact that cows with increased milk production are at higher risk of infection (valckenier).

were greater for cows with an SCC changing increased with increasing SCC

Prior work suggests that although NASM causes elevated qSCC, IMI with NASM likely don’t affect milk yield (cite), although the potential for

Even though it is well-established that … shook … It is yet unclear whether or not the moderate increase in SCC caused by NASM IMI negatively affects milk yield.

Maybe here we say something about better understanding their effect on milk yield; maybe this gets combined with some sort of statement about that

With the exception of larger diagnostic labs and research settings, the best current methods of speciation for NASM (MALDI-TOF, PCR) are not widely available, likely due to a high equipment costs and some technological barriers.

***Language from DAG paper***

2.3. **Case definition, exposure variables and causal diagrams**. The unit of analysis was the individual animal. The out-come of interest was the development of BRD during the first 50 days following induction. The case definition was based on the clinical signs of disease recorded by feedlot staff in computerised hospital records after suspected ill animals were removed from their cohort for examination and treatment.

**Discussion**

Include a section addressing possible biases; simon’s thesis papers provide a good model, good content, and can likely use some of the language from these

Discussion around agnetis and hyicus- Pamela’s 2017 paper (Species Identification and Strain Typing of Staphylococcus agnetis and Staphylococcus hyicus Isolates from Bovine Milk by Use of a Novel Multiplex PCR Assay and Pulsed-Field Gel Electrophoresis); how much did agnetis and hyicus elevate SCC at quarter level, did they act more like contagious or sporadic pathogens; persistent infections vs. transient? Repeat coag- which were positive, which were negative?

*tuf Gene Sequence Analysis Has Greater Discriminatory Power than 16S rRNA Sequence Analysis in Identification of Clinical Isolates of Coagulase-Negative Staphylococci*

16S rRNA and tuf gene sequencing. PCR amplifications were conducted in a total volume of 25 uL containing 2.5 mM deoxynucleoside triphosphates (dNTPs), 10 pmol of each PCR primer, 0.6 U Taq polymerase, 2.5 uL of 10x PCR buffer with 15 mM MgCl2 (Takara Bio, Inc., Shiga, Japan), and 2.5 uL of the template.

The tuf gene was amplified with primers TUF-F (5-GCCAGTTGAGGACGTATTCT-3) and TUF-R (5-CCATTTCAGTACCTTCTGGTAA-3). The PCR conditions for tuf were as follows: 15 min of initial denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C, and 45 s at 72°C, with a final 10-minextension at 72°C.

Gel electrophoresis was used to detect positive PCR signalsand to confirm amplicon lengths of 527 bp for 16S rRNA and 412 bp for thetufgene. Prior to sequencing, the PCR products were purified using the ExoSAP-ITreagent (USB Corporation, Cleveland, OH) according to the manufacturer’sinstructions. Forward and reverse sequencing reactions were conducted for eachof the amplified products. The sequencing reaction mixture for 16S rRNA con-sisted of 10l of MicroSeq 500 sequencing mix (containing 1.6 pmol of MSQ-For MSQ-R) primers, 2.9l of molecular-grade water, and 1l of the purifiedPCR product. For thetufgene, sequencing reactions were performed usingBigDye Terminator, version 3.1, reagents (Applied Biosystems Inc., Foster City,CA). Briefly, the sequencing reaction mixture consisted of 1l of BigDye ReadyReaction mix, 3.5l of BigDye sequencing buffer (5) (Applied BiosystemsInc.), 1.6l of a 1-pmol primer, 2.9l of molecular-grade water, and 1lofthepurified PCR product; the final reaction volume was 10l. The thermal cyclingconditions were as follows: 25 cycles of 10 s at 96°C,5sat50°C, and 4 min at60°C. The sequencing products were purified using ethanol–sodium acetate.Sequencing reactions were performed on an ABI Prism 3130xl genetic analyzer(Applied Biosystems Inc.) according to the standard automated sequencer pro-tocols

Confirmation of species identification by housekeeping gene sequence analysis.

M and m from pamela’s agnetis/hyicus paper

A single colony was picked from CBA and inoculated into 100 μl of 1× Tris-EDTA (Promega, Madison, WI, USA) for lysate preparation. Standard PCR, with the addition of an initial denaturation step of 15 min at 94°C, was used to amplify the *rpoB* ([18](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5442534/#B18)) and *tuf* ([22](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5442534/#B22)) genes. The PCR for *rpoB* gene identification and subsequent sequencing for species identification were done on all isolates, and the PCR for *tuf* gene identification and subsequent sequencing were conducted only on isolates not definitively identified as *S. agnetis* or *S. hyicus*. The resultant PCR products were purified using a commercial kit (Invitrogen, Carlsbad, CA, USA) and submitted to the University of Missouri DNA Core Facility for Sanger sequencing. Sequences were compared to the GenBank database using the publicly available nucleotide-BLAST algorithm ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov/)). For *rpoB* analysis, species identity was confirmed if the sequence had ≥97% identity with a single species of *Staphylococcus* in the GenBank database ([34](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5442534/#B34)). For *tuf* gene analysis, species were confirmed if sequence identity was ≥98.0% with >0.8% separation between species ([22](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5442534/#B22)).