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Genetic Resistance to *Staphylococcus aureus* Mastitis Associated with Bovine CXCR1

In partial fulfillment of the

Requirements for the

Chancellor's Honors Program

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Abstract

Identifying genetic markers for resistance to *Staphylococcus aureus* in dairy cattle can make producers more efficient in cow selection and increase the shelf life of milk through decreased bacterial presence. CXCR1 is associated with inflammation and has been identified as a potential genetic marker for resistance to this pathogen. My hypothesis states there is an association between CXCR1 single nucleotide polymorphisms (SNPs) and resistance to *S. aureus*-caused mastitis.

DNA was isolated from 130 cows at Middle Tennessee Research and Education Center, half of which tested positive for the presence of *S. aureus.* CXCR1 was amplified and samples were sequenced to pinpoint previously identified SNPs: +621, +735, +816, and +980. The association between these SNPs and infection rate was evaluated to determine if a relationship existed between CXCR1 and *S. aureus* resistance. The top three tag SNP haplotypes accounted for 89.26% of the samples. Out of seven haplotype combinations for both alleles, the top two accounted for 50.87% of the samples. For subclinical data, two haplotype combinations appeared to be statistically more susceptible to *S . aureus*, (p-value<0.05). The clinical data did not result in a significant difference in resistance among haplotype combinations.

Introduction

Mastitis, or inflammation of the mammary gland, negatively impacts animal health, productivity, and milk quality and is a major cost to dairy producers. This disease accounts for almost $1.7-2 billion dollars annually[1]. Clinical mastitis presents with abnormal milk and hot, inflamed mammary glands, while subclinical mastitis cannot be detected visually.

Regardless, a high Somatic Cell Count (SCC), or the white cell count, in milk may detect infections of both forms. Upon introduction of mastitis-causing pathogens, leukocytes enter the infected tissue from the blood, thus raising the SCC observed in the sample. Identifying genetic markers for mastitis in dairy cattle can make producers more efficient and increase shelf life of milk through decreased bacteria presence and SCCs.

One of the key contagious pathogens that causes mastitis is *Staphylococcus aureus,* a common cause of subclinical mastitis. *S. aureus* is a predominant mastitis pathogen found on farms, difficult to detect in a timely fashion after infection, and is a highly contagious pathogen transmitted during the milking process. Unfortunately cure rates are less than 30% after antibiotic therapy. Thus, once a cow contracts S*. aureus* caused mastitis and antibiotic therapy has proven ineffective, removal from the herd is recommended. The ability to select for cows resistant to this bacteria is of great importance. This study will focus on this key pathogen.

An association may be found between *S. aureus* resistance and candidate genes located in previously identified regions of the chromosome. Of particular interest is CXCR1, a receptor for interleukin 8 that has been associated with calcium signaling, adhesion molecule expression, and neutrophil activity[2]. The relationship between *S. aureus* and CXCR1 will be tested at single nucleotide polymorphisms +621, +735, +816, and +980. My hypothesis states there is an association between CXCR1 single nucleotide polymorphisms (SNP's) and resistance to *Staphylococcus aureus*-caused mastitis.

Materials and Methods

Cow Selection

Blood samples from 120 cows were selected from the University of Tennessee Middle Tennessee Research and Educational Center (MTREC; Spring Hill, Tennessee). Sixty samples came from cows with known cases of mastitis with *Staph. aureus* present in milk samples. The other sixty samples represented cows with no recorded instances of mastitis involving *Staph. aureus.* All samples were taken during the same time period between 2002-2004. Samples were collected from cows exhibiting clinical signs of infection. Additional samples were collected every three to four months to identify subclinical infections. Samples were collected using aseptic techniques, following guidelines from the National Mastitis Council.

DNA Isolation

To isolate and extract DNA from whole blood samples, UltraClean BloodSpin DNA Isolation by MoBio Tech was utilized. 200 µL of whole blood was mixed with 10 µL Proteinase K with a concentration of 50 µl/ml to break up proteins, followed by the addition of 200 µL lysis reagent of chaotropic salt and a detergent. The samples were incubated at 65°C for ten minutes then cooled to room temperature to ensure complete protein denaturation and cell lysis. 200 µL of 100% ethanol, which promotes DNA binding, was thoroughly mixed with the samples. The lysate was transferred into the spin filter and centrifuged at 14,500 rpm. This utilized the principle of DNA binding to the silica membrane filter in high salinity conditions while excluding denatured protein, RNA, and other waste. 500µL of a salt-based wash was added to the filter to clean the DNA and was centrifuged. Then 500 µL of an ethanol wash was added to clear residual salt contaminants. The filters were dried before two 100 µL additions 10 mM Tris (heated to 70°C), 1 mM

EDTA were added to the filter and centrifuged. The DNA is eluted off the filter in the absence of salt.

DNA quantification

DNA quantification is a necessary measure to ensure the quality of the isolated DNA prior to its dilution and use in Polymerase Chain Reaction procedures. Isolated DNA samples were tested for concentration and purity using a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Fisher Waltham, MA). 1.5 µL of each sample was loaded onto the stage to obtain ng/µL and A260/A280 ratio. The minimum concentration required to sequence DNA with current technology is 10 ng/µL. The A260/A280 ratio compares the absorption of nucleic acids (A260) and the absorption of proteins (A280). Generally, this ratio is desired to be between 1.7 to 2.0.

Primer Design

Primers were derived for the amplification of CXCR1 using Primer3 (http://frodo.wi.mit.edu). Primers must be designed to ensure the desired part of genome, in this instance CXCR1, is amplified. The forward primer is the direct complement of approximately 20 base pairs at the 5' end of CXCR1, while the reverse primer must complement the 3' end of CXCR1. The two primers should have a similar melting temperature for an optimal PCR product. With the designed primers, the PCR product should be approximately 900 base pairs. Primers were checked for specificity and the product was sequenced to very the correct product was obtained.

	Bases	TM (Celcius)	Sequence Name	Sequence
P236	26	59	Bt_4-226_out F	CTT CAT CTT CCG TGA GGC CTA TCA AC
P237	24	63	Bt_4-226_out R	AAG GGA AGG GGA CTT TCC TGG CTG

PCR

The PCR Mastermix utilized was GoTaq Green Master Mix M712b (Promega; Madison, WI). At 8.5 pH and 1.5 µM, it contains DNA polymerase, dNTPs–building blocks for the new DNA strands, and $MgCl_2$, along with blue and yellow dyes for electrophoresis. For each sample, 12.5 µL of Mastermix was combined with 1 µL of 10x diluted DNA (ranging from 34.73 ng/µL to 140.81 ng/µL), 1 µL of combined primers [100 µM] p236 and p237, and 10.5 µL of nuclease free water. Each sample was loaded individually into 0.2mL polypropylene PCR tubes.

The samples were placed into a thermocycler that performs the PCR process. An initial 94°C step occurred for two minutes to hot start the polymerase. The denaturation step followed, 94°C for fifteen seconds, to break apart the double stranded DNA. Then annealing occurred by holding the samples at 62°C for 15 seconds so the primers could hybridize with the DNA. It should be noted the annealing temperature should be only a few degrees below the melting temperature of the primers. Finally, the temperature was raised to 72°C for one minute for the elongation phase. In this step, the polymerase uses dNTPs to build a complement to the template primer-DNA strand. These three key steps,

denaturation, annealing, and elongation, are repeated by the thermocycler 45 times before the final elongation step at 72°C for 10 minutes. At this point the PCR reaction is complete and the samples are held at 4°C until the samples are used or placed in the freezer for long-term storage.

Electrophoresis

Electrophoresis was used to determine the presence of the correct PCR product for each sample. This was performed using a 1% agarose gel prepared by combining and boiling 50 mL 1x TAE buffer and 0.5 g NuSieve 3:1 Agarose (Fisher Molecular Biology, PA). After the mixture cooled for ten minutes, 5 µL of ethidium bromide was added and the solution was poured into the electrophoresis mold to solidify.

5-10 µL of each sample were loaded into one of the wells in the gel. Smaller fragments travel further down the gel while larger DNA fragments remain closer to the wells. Electrophoresis was conducted at 80 volts for 45 minutes (Bio-Rad Laboratories, CA). The gels were then viewed using a Fluor Chem 5500 ultraviolet light cabinet (Alpha Innotech, CA) to ensure the proper PCR product was obtained. This works by using ultraviolet light to visualize where the bands on the gel occur. A band at approximately 900 base pairs indicated the presence of the appropriate product when compared to HyperLadder 1kb (200-10,037 bp, Biolab, GA).

Purification of PCR Products

After verifying the proper PCR product was obtained, the amplified DNA sequences were purified to remove excess primers and dNTPs, then concentrated using DNA Clean and Concentrator-5 (Zymo Research Corp., Orange, CA). 20 µL of each sample was combined with 80 µL of nuclease-free water. 200 µL of binding buffer was thoroughly

mixed in and centrifuged through a spin filter. This enabled the DNA to bind to the filter. This was followed by two additions of 200 µL of wash buffer and centrifugation. The wash buffer worked to remove any other products that may have adhered to the filter. Finally, two 10 µL additions of nuclease-free water were added to the spin filter and centrifuged. Thus 20 µL of purified, concentrated DNA specific to the primers was isolated.

DNA Sequencing

Following purification, the amplicon sequences were sent to the University of Tennessee-Knoxville's Molecular Biology Resource Facility in Walters Life Sciences Building. Sequence chromatograms were received via email and evaluated to determine if SNP's were present at 4 locations in CXCR1: +621 (G>A), +735(G>C), +816(A>C), and +980(G>A)[3].

Staphylococcus aureus Infection Rate

Every sample had previously recorded information on the quantity and type of bacteria present in each quarter of the udder for each test date. Each cow with *S. aureus* present in one or more quarters was identified. Next, the number of days between the first and last test dates for each cow was determined (total time). To determine the average number of *S. aureus* infections per a 305 day lactation, the number of separate incidences of *S. aureus* was divided by the total time and multiplied by 305. This gave a standardized rate of infection for all samples.

$$\frac{\text{Number of } S.\ aureus \text{ infections}}{\text{Total Time}} \times 305 = \text{Standardized Infection Rate}$$

Cows that tested positive for *S. aureus* and presented clinical signs of mastitis (red, hot, inflamed mammary gland, abnormal milk, etc.) during the sample period were classified as "clinical". Cows that tested positive for *S. aureus* during the sample were categorized as "subclinical" regardless of clinical signs. Therefore, cows may be classified as both clinical and subclinical. It should be noted cows may have tested positive for other types of bacteria during that time.

Five clinical cows (112, 545, 3068, 775, and 863) were culled after only one sample collection. Therefore, we operated under the assumption these cows had one incidence of mastitis for a 305 day lactation. A subset of 66 cows were analyzed using a mixed ANOVA model in SAS.

Results

The average frequency of each genotype was calculated for each SNP, and allele frequencies were derived from the genotype frequency by adding the appropriate homozygous genotype frequency and half of the heterozygous genotype frequency. Haplotypes were compiled and labeled according to a numbering system previously in place in the laboratory. The frequencies for tag SNP haplotypes and amino acid haplotypes as shown in Table 1.

Table 1- Single nucleotide polymorphism and amino acid haplotypes present in population and their relative frequencies.

Tag SNP Haplotypes	Frequency	AA Haplotypes	Frequency
+621 +735 +816 +980		365-621-735-980-955 122-206-245-327-332	
GCCA	25.62%	TGCAA	26.03%
GCAA	0.41%	VWHKH	
AGCG	0%	CAGGG	0.00%
		AX	
GCAC	19.01%	TGCGG	19.84%
GCCG	0.83%	VWHRR	
GGAG	44.63%	AWQRR	48.76%
GGCG	4.13%	CGGGG	
GGCA	0.83%	CGGAA	0.83%
		AWQKH	
	n=121		n=121

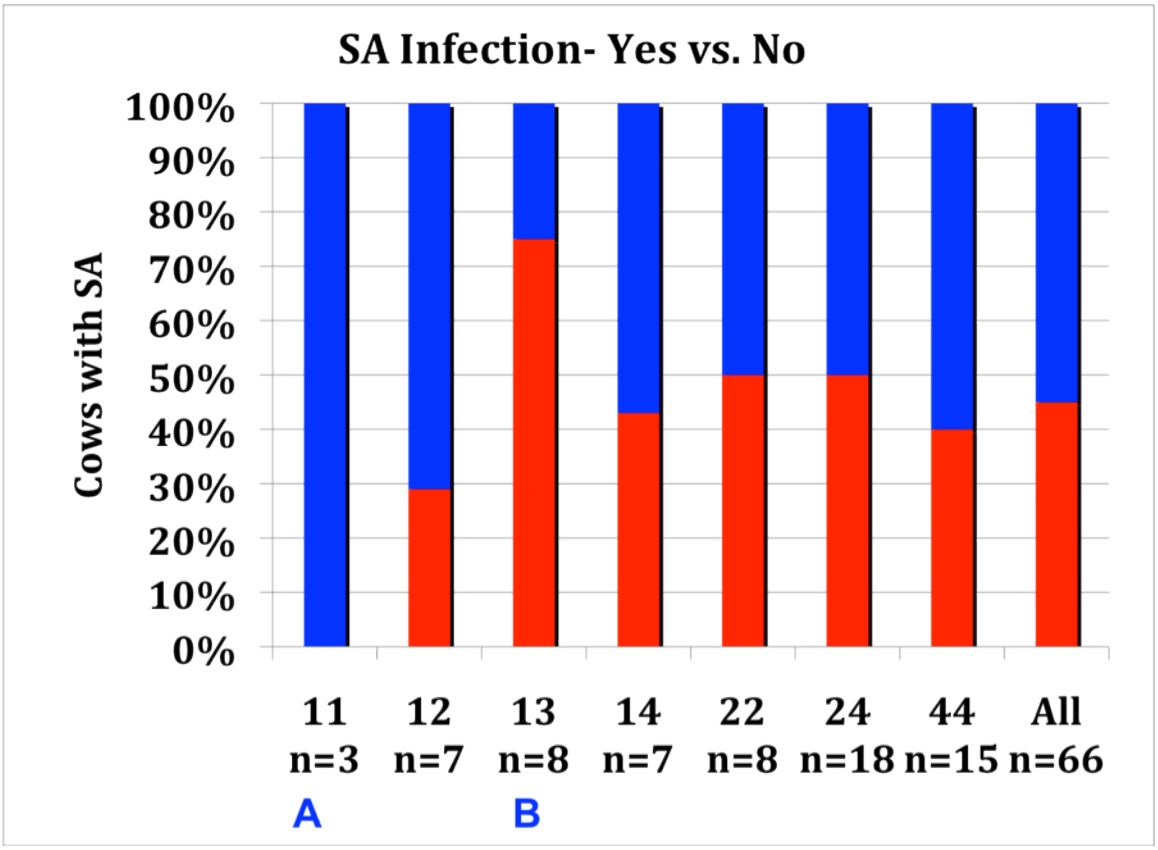
The top three tag SNP haplotypes (GCCA, GCAC, and GGAG) accounted for 89.26% of the samples. It should be noted no samples were found to have tag SNP AGCG, as none of the sampled population had adenine at position +621. The top two amino acid haplotypes (AWQRR and VWHKH) contributed to 74.79% of the sample population. The remainder of the population was composed of VWHRR and AWQKH. AX was not represented in the sampled population.

Table 2- Amino acid combinations were assigned a haplotype combination number, and relative frequencies were calculated for each.

Assigned Haplotype Number	Amino Acid Combination	Frequency (%)	Number Cows (Subclinical and Clinical)
11	VWHRR x VWHRR	2.63%	3
12	VWHKH x VWHRR	13.16%	15
13	VWHRR x AWQKH	8.77%	10
14	VWHRR x AWQRR	15.79%	18
22	VWHKH x VWHKH	8.77%	10
24	VWHKH x AWQRR	23.68%	27
44	AWQRR x AWQRR	27.19%	31

For the entire population, haplotype combinations 44 and 24 were the most dominant, accounting for 50.87% of the sample population (Table 2). Haplotype combinations 12 and 14 accounted for greater than 10% of the population as well. Only haplotype combination 11 accounted for less than 5% of the population, with a population size of three cows.

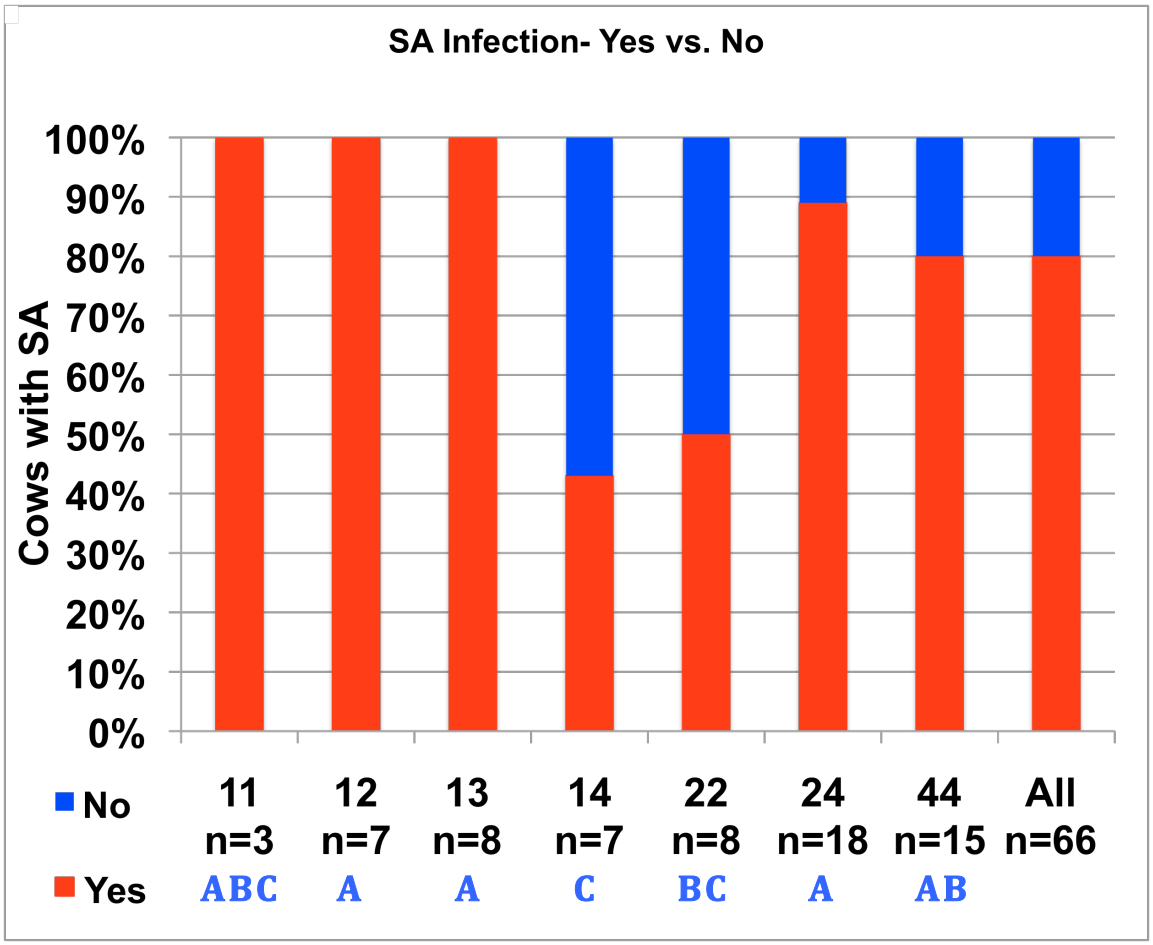
Figure 1- The yes/no analysis of susceptibility to *S. aureus* per haplotype combination for ***clinical*** mastitis. Red shows the percent susceptible *to S. aureus*. Blue indicates no *S. aureus* infection. A and B denote statistical differences between haplotype combinations.



The clinical data showed an average 40% susceptibility to *S. aureus* across all haplotype combinations (Figure 1). Haplotype combination 11 showed no cows with clinical *S. aureus* infections, while combination 13 was 75% susceptible. An ANOVA test ran with the CXCR1 haplotype combination equal to the treatment group demonstrated the percentage of mastitis was similar between groups. Chi-Square analysis also showed similar percentage of mastitis among groups (p= 0.3658). A trend for specific comparisons, defined as a p value less than 0.10, was found for combinations 11 vs. 24 and 12 vs. 13.

Combinations 13 vs. 44 was close to this level, at p= 0.11. A significant difference ($p<0.05$) was found for combinations 11 vs. 13. Generally, combinations 11 and 12 had low mastitis prevalence while combinations 14, 24, and 44 were average. Combinations 22 and 13 had the greatest prevalence of mastitis.

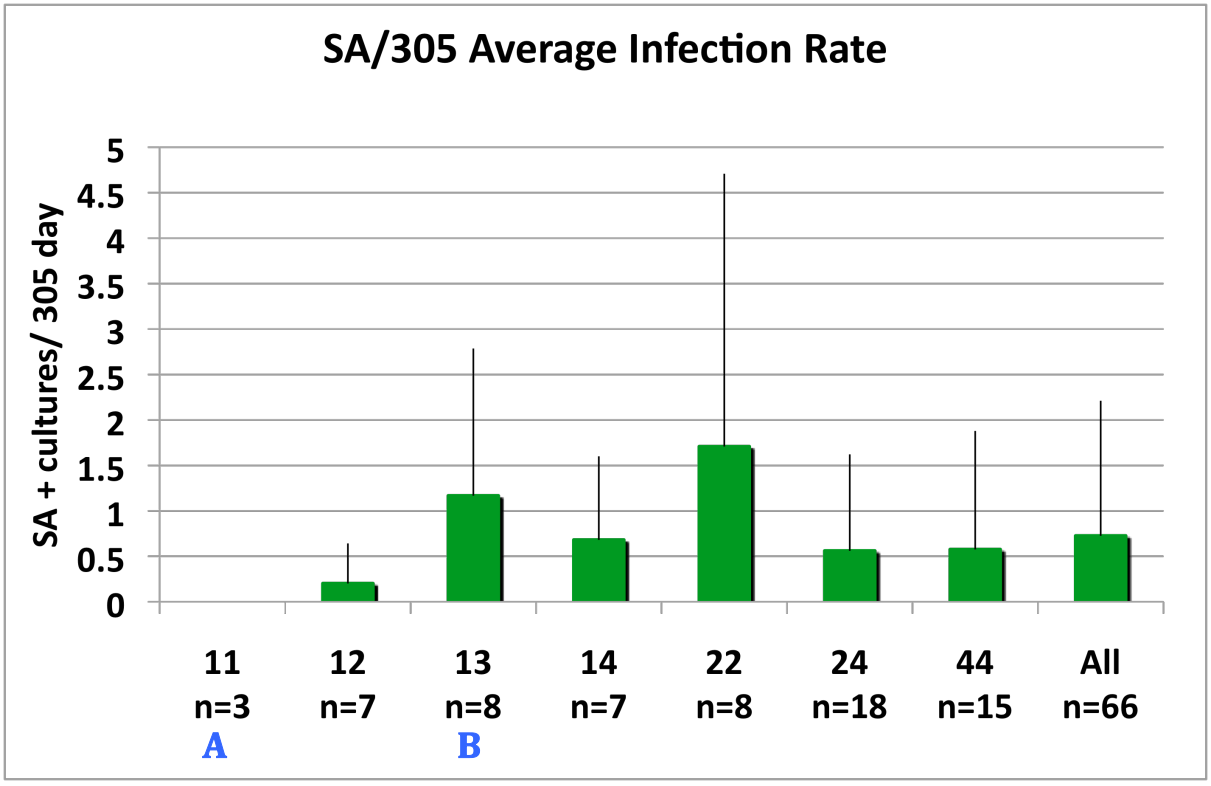
Figure 2- The yes/no analysis of susceptibility to *S. aureus* per haplotype combination for ***subclinical*** mastitis. The red portion of the column shows the percent with *S. aureus* infections, while the blue shows the percentage without *S. aureus* infections.



For subclinical *S. aureus* mastitis, 80% of the cows in the sample population were susceptible (Figure 2). Haplotype combinations 14 and 22 deviate from the average 80:20

ratio, having 42% and 50% susceptibility to *S. aureus*, respectively. Every cow with haplotype combinations 11, 12, and 13 tested positive for subclinical *S. aureus* infections. The Chi-Square p-value for all haplotypes was found to be 0.0132. Specific comparisons were made between each haplotype combination. A "trend," defined as a p value of less than 0.10, was noted for combinations 11 vs. 14 and 14 vs. 44. Significant differences between two haplotype combinations, determined by a p value less than 0.05, were found for the following combinations: 12 vs. 22, 12 vs. 14, 13 vs. 22, 13 vs. 14, 14 vs. 24, and 22 vs. 24. These specific comparisons helped distinguish between susceptibility groups A, B, and C. The "A" group trends toward susceptibility, while "C" has the greatest resistance.

Figure 3- The average number of *S. aureus* ***clinical*** mastitis cases per 305 day lactation for each haplotype. Combination 22 had the greatest infection rate but no differences were found to be significant between haplotypes. Raw means are shown in green, with standard error denoted by black lines.



To compare the rate of clinical or subclinical mastitis between CXCR1 haplotypes, the average number of infections per haplotype combination was calculated by dividing the total number of infections for each cow and dividing by 305 (number of days in one lactation) to standardize the duration. The error bars represent the standard error. For the clinical mastitis, haplotype combination 22 showed the greatest infection rate (1.70) and the most variability (3.00) (Figure 3). Combination 11 showed no clinical infections, although the small sample size should be noted. Generally, combinations 11 and 12 had low infection rates while combinations 14, 24, and 44 were average. Combinations 22 and 13 had the greatest infection rates.

Figure 4- The average number of *S. aureus* ***subclinical*** mastitis cases per 305 day lactation for each haplotype combination. The raw mean is shown in green and black lines above the bars present standard error of the mean. Statistical differences among the groups are denoted by A, B, and C.

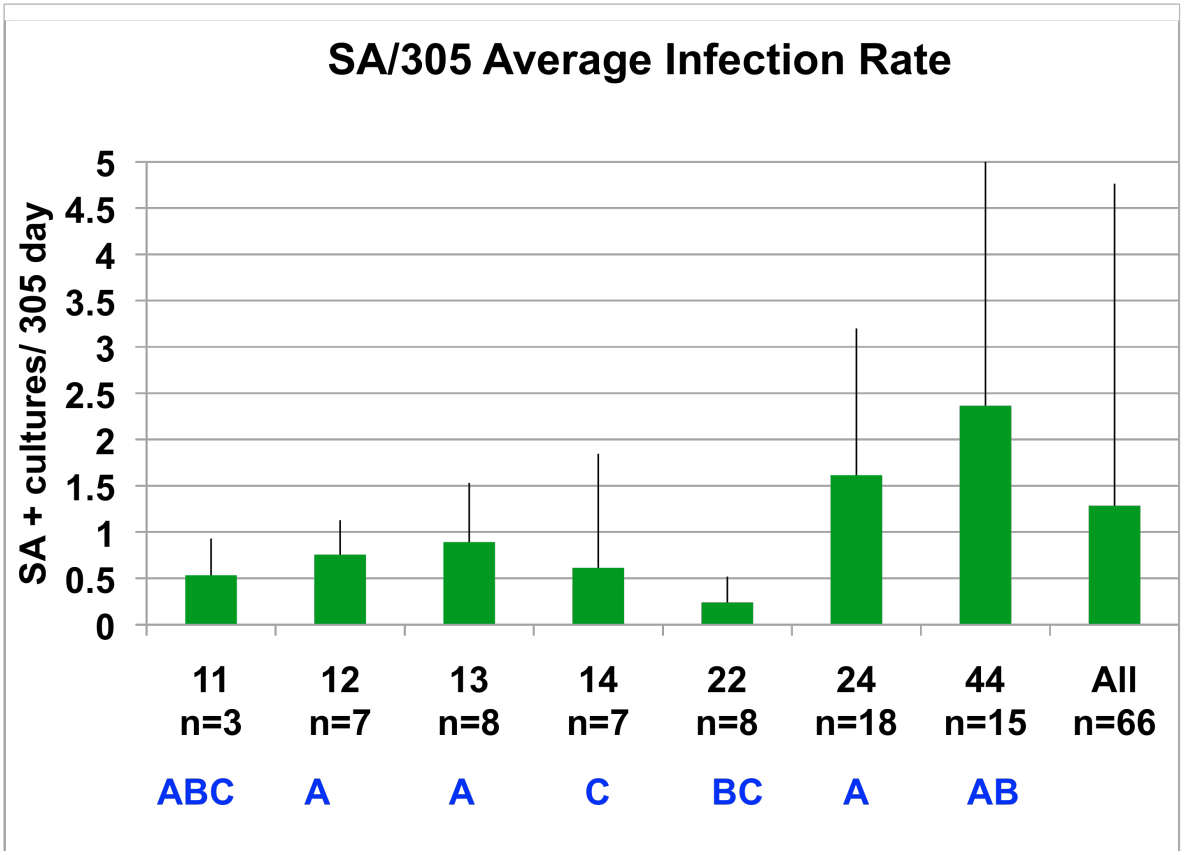


Figure 4 shows the average number of *S. aureus* infections classified as subclinical mastitis. The raw mean for each haplotype combination was then calculated and graphed. Haplotype combination 44 had the highest number of infections, averaging 2.36 infections per lactation. This combination also had the greatest standard error, measuring 7.04. Haplotype combination 22 reported the lowest rate of infection, averaging 0.24 infections per lactation. The average for the sample population was 1.28 infections per lactation.

Discussion

The purpose of this study was to compare and determine if particular SNPs in CXCR1 could indicate resistance to *S. aureus* infections. A comparison was made of haplotype frequencies with previous papers on CXCR1. In Dr. G.M. Pighetti's "The bovine CXCR1 gene is highly polymorphic," VWHKH comprised 32% of the population, compared to 26% for this study [4]. Similarly, VWHRR accounted for 19% in both populations. However, a discrepancy was seen with AX; the frequency of AX in Pighetti and colleagues (2012) population equals 20%, while no cows were found with the corresponding tag SNP haplotype (AGCG) in this study. AWQRR accounted for 18% of Pighetti and colleagues (2012) population, but it accounted for a much greater percentage, 48%, in this population. These discrepancies may be due to the selection process for each study. Pighetti and colleagues (2012) population cows were selected at random; in contrast, cows were specifically chosen in this study for the presence or absence of *S. aureus* mastitis.

Likewise, a comparison of haplotype combinations was made with "Bovine mammary interleukin-8 receptor expression and genetic association with *Streptococcus uberis* based mastitis." by Siebert[5]. Combination 11 showed the most discrepancy between populations, representing 13% of Siebert's population and only 2.6% of this study's.

Haplotype combinations 13 and 22 were not present in Siebert's population. However, the remainder of the combinations were very similar between populations. Combination 12 represented 13% and 18% of Siebert's and this study's population, respectively. Combination 14 accounted for 15% of both populations. Similarly, combination 24 represented 23% of both populations. Combination 44 accounted for 27% of Siebert's population and 28% of the current study's. Again, variation can be accounted for through the selection process. Siebert's sample population of second and third lactation cows was selected for low frequency of Johnes, Brucellosis, BLV, and low titers against *S. uberis*.

Pighetti and colleagues (2012) identified SNP location +735 (C>G, H245Q) as a part of linkage group 1. C alleles at this location, represented by the third letter of the haplotype, were shown to be more susceptible to mastitis[6]. This corresponds to VWHKH and VWHRR, haplotypes 1 and 2. For this study, haplotype combinations 12, 13, and 24 have greater susceptibility than combinations 14 and 22 according to the subclinical data. Thus, combinations 12 and 24 have data consistent with the previous paper, but other combinations do not reflect the trend. Interestingly, combination 22 is more resistant to *S. aureus* infections.

Subclinically, combination 13 (CG at +735) had a 100% of cows infected with average infection levels. Combination 24 (CG at +735) had a high incidence of infection, as well as a high standardized infection rate. Conversely, combination 14 (also CG at +735) had an average infection level with only a small proportion of cows susceptible to *S. aureus.* It is statistically more resistant to subclinical *S. aureus* mastitis than other haplotype combinations. +735 heterozygotes as a whole tend to have *S. aureus* infections, but results are not entirely consistent.

Combination 22(CC) had low subclinical infection rates as well as *S. aureus* levels. Statistically it is consistently more resistant to subclinical *S. aureus* mastitis. 100% of combination 12 cows (CC at +735) had subclinical *S. aureus* infections, although low *S. aureus*/305 day levels. This may indicate cows are initially susceptible but also efficient at fighting off the infection. Combination 11 (CC at +735) had a sample size of only 3, making it difficult to make assumptions about susceptibility; subclinically, all cows were infected but had low infection levels. Combination 44 (GG at +735) had the greatest variation and is accordingly close to average, bearing similarity to both susceptibility groups A and B.

Clinically, only haplotype combinations 11 and 13 were statistically different. Combination 13 had the highest proportion of infected cows, while combination 11 had no cows with *S. aureus.* However, firm conclusions about combination 11 cannot be made at this time due to a small sample size, although it does suggest potential for resistance.

At position +735, C>G results in a change from histidine to glutamine at position 245. Previous studies have shown CC and CG genotypes have decreased neutrophil activity, including migration, compared to GG at +735, possibly due to the associated amino acid change.[7] Neutrophil levels in blood and milk were not directly monitored in this study, but this could allude to why certain genetic backgrounds have added resistance to *S. aureus.* Lower neutrophil levels with CC at +735 could explain the association between haplotype combination 12 and *S. aureus* susceptibility. Heterozygotes at +735 vary in results, as seen with haplotype combinations 13, 14, and 24; combinations 13 and 24 have higher infection rates while combination 14 appears to be more resistant. Haplotype combination 44 (GG at +735) is of particular interest; it accounts for the largest percentage of the entire population and has an infection rate understandably close to average. However, it has a

high *S. aureus/*305 day infection level, contradicting previous results that GG is more resistant. Similarly, there is contradiction with combination 22, which is CC at + 735 but has a low incidence of *S. aureus*.

Overall, two haplotype combinations, 24 and 44, represented the majority of the population. For subclinical data, haplotype combinations 14 and 22 were found to be statistically more resistant to *S. aureus.* The clinical data did not show differences among haplotypes, although combination 11 could potentially be more resistant to *S. aureus* infection. Future studies may be done to further investigate the mechanisms of resistance for haplotype combinations 14 and 22. Moreover, further examination of haplotype combination 11 could yield more definitive results by increasing sample size.

Although the H245Q SNP at nucleotide position +735 has been emphasized, the other SNPs may also have an effect on a cow's immune response. For example, the conserved amino acids KH (positions 327-332) have been associated with binding of LASP-1/adaptin-2 and internalization of the receptor[8]. Therefore, the +735 SNP is not the only SNP than can potentially influence functionality of the entire receptor and it is the combination of SNPs within a protein that determines overall function. Furthermore, the environment was the same for all cows in the study, but there may be other factors impacting mastitis, which includes the remaining genetic background not accounted for in this study.

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