Association of CXCR2 Polymorphisms with Subclinical and Clinical Mastitis in Dairy Cattle

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ABSTRACT

The ability to identify objectively cows that are more or less susceptible to mastitis has been a long-standing goal. Genetic markers associated with inflammatory responses during mastitis could aid in selection of these cattle. One potential marker is CXCR2, a chemokine receptor required for neutrophil migration to infection sites, which contains single nucleotide polymorphisms (SNP) within the gene. The objective of this experiment was to evaluate the association of CXCR2 SNP genotypes with subclinical and clinical mastitis. Thirty-seven Holstein and 42 Jersey cows that completed at least 2 full lactations were used. Quarter foremilk samples were collected for bacteriological examination quarterly and when cows exhibited clinical mastitis. Subclinical mastitis was defined as the presence of the same pathogen in the same quarter in at least 2 of 3 consecutive samples. A significant association was detected between CXCR2 SNP +777 genotype and percentages of subclinical mastitis cases in Holsteins. Holsteins expressing genotype GG had decreased percentages of subclinical mastitis, but genotype CC cows had increased percentages of subclinical mastitis. Significant differences in clinical mastitis incidence were not detected between genotypes for either breed. This approach of genetically identifying mastitis resistant cows may represent an effective means of marker-assisted selection for mastitis and other inflammatory diseases involving neutrophils.

(Key words: mastitis, single nucleotide polymorphism, CXCR2, interleukin-8 receptor)

Abbreviation key: BTA = *Bos taurus* autosome, DES = Dairy Experiment Station, MTES = Middle Tennessee Experiment Station, SNP = single nucleotide polymorphism.

INTRODUCTION

Mastitis continues to be the most economically devastating disease affecting the dairy industry. Identifi-

cation of one or more genetic markers associated with mastitis susceptibility, resistance, or both would allow producers to decrease costs associated with mastitis by improving herd health through animal selection. Because mastitis and other infectious diseases are complex and use many different genes, it is highly probable that several genetic markers used in combination would provide the best means for making genetic progress. Genes associated with immune responses of the mammary gland are potential markers because of their importance in mastitis. Several studies have identified polymorphic bovine genes responsible for encoding the major histocompatibility complex, cytokine and cytokine receptors, and natural resistance associated macrophage protein-1 (Dietz et al., 1997; Grosse et al., 1999; Ables et al., 2002). However, only a few studies have associated major histocompatibility complex alleles DRB3.2 *3, *11, *16, and *23 with mastitis incidence or immune function (Dietz et al., 1997; Kelm et al., 1997; Sharif et al., 1998, 2000; Park et al., 2004).

Genes associated with neutrophil function are potential genetic markers for mastitis, as neutrophil migration from blood to the site of infection is essential for resolution of most mastitis pathogens (Paape et al., 2000). The ability of neutrophils to migrate into infected tissues is dependent upon recognition of inflammatory mediators by neutrophil cytokine, chemokine, and complement receptors (Burvenich et al., 1994). Two chemokine receptors present on neutrophil surfaces, CXCR1 and CXCR2, are required for maximum neutrophil function during infection (Murphy and Tiffany, 1991). Recognition of chemokines by CXCR1 and CXCR2 induces neutrophil activation, chemotaxis, and eventual phagocytosis of pathogens (Peveri et al., 1988; Podolin et al., 2002).

Both CXCR1 and CXCR2 are potential candidate genes for mastitis resistance; however, only the sequence for bovine CXCR2 mRNA is publicly available (GenBank Accession No. U19947). The bovine CXCR2 gene has been mapped to BTA (*Bos taurus* autosome) 2, approximately 90.3 cM from the centromere. This region on BTA 2 shares conserved synteny and gene order with both *Homo sapien* autosome 2 and mouse

Received December 11, 2003.

Accepted April 2, 2004.

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Table 1. Single nucleotide polymorphism (SNP) +777 genotype frequencies within the CXCR2 locus of Holstein and Jersey dairy cattle.

	SNP genotype	Jersey frequency (n = 42)	Holstein frequency (n = 37)
777 ¹	GG	0.76	0.24
	GC	0.22	0.65
	CC	0.02	0.11

¹Genotype frequencies were affected by breed ($P < 0.05$).

chromosome 1 (Sonstegard et al., 1997; Grosse et al., 1999). Single nucleotide polymorphisms (SNP) have been identified within the human CXCR2 gene and have been shown to vary in different geographical populations (Kato et al., 2000; Renzoni et al., 2000). Several of these SNP have been associated with susceptibility to inflammatory diseases. In an effort to expand the pool of genetic markers that could potentially be associated with disease in cattle, Grosse et al. (1999) evaluated the presence of polymorphisms within a variety of immune-related genes, including the coding region of CXCR2 present within the third exon. Within this region, 4 SNP were identified that varied only in certain parents, and their alleles separated according to Mendelian inheritance patterns in their progeny. Further analysis of the Meat Animal Research Center cattle reference population revealed the presence of these SNP in Angus and Hereford pure and crossbred cattle (Heaton et al., 2001). To the best of our knowledge, neither the frequency of these SNP nor their association with disease susceptibility has been evaluated in the general bovine population.

Examination of the CXCR2 sequence in Holstein and Jersey dairy cattle revealed one additional SNP within a 311-bp segment of the coding region not present in the beef cattle population. All 5 identified polymorphisms exhibited strong linkage disequilibrium ($P < 0.05$) and were located at positions +612, +684, +777, +858, and +861 relative to the published sequence (GenBank Accession No. U19947; Youngerman et al., unpublished, 2003). As might be expected, the frequency of these polymorphisms varied between breeds, as demonstrated by SNP +777 in Table 1. Four of the identified SNP are synonymous polymorphisms and do not affect the amino acid sequence of CXCR2 (+612, +684, +858, +861). However, the nonsynonymous (G to C) SNP at +777 results in a glutamine to histidine substitution at amino acid 245 (Youngerman et al., unpublished, 2003). Amino acid 245 is located within the third intracellular loop of CXCR2, an important region involved in mediating calcium signaling and mobilization as well as G-protein binding when stimulated by interleukin-8. Therefore, the bovine CXCR2

gene is an excellent potential candidate marker for mastitis, as CXCR2 is a critical component during neutrophil migration to the mammary gland during mastitis; CXCR2 polymorphisms have been associated with disease susceptibility in humans, and several polymorphisms have been identified in cattle. The objective of this experiment was to determine whether individual SNP or a combination of the 5 SNP (i.e., haplotype) within the bovine CXCR2 gene were associated with subclinical mastitis, clinical mastitis, or both in Holstein and Jersey cattle.

MATERIALS AND METHODS

Animal Selection

Forty-two Jersey cows from the Dairy Experiment Station (DES; Lewisburg, TN) and 37 Holstein cows from the Middle Tennessee Experiment Station (MTES; Spring Hill) that had at least 2 full lactations were selected at random. The Jersey cattle population at the DES had approximately 7.8% inbreeding, which is comparable to the general Jersey population (AIPL, 2004). Although the actual inbreeding value of the MTES herd is not known, approximately 50% of the herd was composed of cows purchased from outside sources and was expected to mimic the 4.9% inbreeding level found within the general Holstein population in the US (AIPL, 2004). The majority of Jersey cows completed at least 4 lactations; Holstein cows completed at least 3 lactations. These animals were genotyped previously by sequencing a 311-bp fragment of the CXCR2 gene amplified from genomic DNA and inserted into a TOPO vector for cloning (Youngerman et al., unpublished, 2003).

Experimental Herds

The DES research herd in Lewisburg, Tennessee consists of approximately 170 lactating Jersey cows. Cows were milked twice daily in a 12-stall trigon milking parlor equipped with automatic milking machine takeoffs (Surge; Babson Bros., Oak Brook, IL). The MTES dairy research herd in Spring Hill, Tennessee was composed of approximately 150 lactating Holstein cows. Lactating cows were milked twice daily in a double-eight parallel parlor equipped with automated milking machine take-offs (DeLaval, Kansas City, MO). Milking machines were backflushed (Surge Backflush II; Babson Bros.) with water following cow removal. Milking equipment was evaluated routinely and maintained by manufacturer's recommendation. Pre- and postmilking teat disinfection was performed. Cows were pastured 4 to 6 h/d, weather and pasture permitting. Cows were housed in free stalls bedded

with separated dairy waste solids from a manure slurry (Alfa-Laval, Inc., Kansas City, MO) at the DES herd and with sawdust at the MTES herd. All cows were dried off approximately 6 to 8 wk before expected calving. An antibiotic preparation approved for use in nonlactating cows was infused into all cows' mammary glands following the last milking of lactation.

Bacteriological Examination of Foremilk Samples

One foremilk sample from each quarter was collected from all lactating cows in both herds approximately every 3 mo, prior to dry off, 1 wk after calving, and when cows exhibited clinical mastitis and were evaluated microbiologically. Prior to sample collection, teats of cows were cleaned and dried with individual disposable paper towels, and teat ends were sanitized with swabs containing 70% isopropyl alcohol. After sample collection, samples were stored frozen at -20°C until transported to the laboratory. Milk samples were examined following procedures recommended by the National Mastitis Council (Hogan et al., 1999). Samples of mammary secretion ($10\ \mu\text{L}$) from each mammary gland were plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Becton Dickinson and Company, Franklin Lakes, NJ). Plates were incubated at 37°C , and bacterial growth was observed and recorded at 24-h intervals for 3 d. Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics, Gram stain reaction, and catalase test. Isolates identified presumptively as staphylococci were tested for coagulase production by the tube coagulase method. Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin and sodium hippurate, and CAMP reaction. Streptococcal organisms were identified to the species level using the API 20 Strep system (bioMérieux Inc., Durham, NC) and a streptococcal agglutination system (Streptex, Remel, Lenexa, KS). Gram-negative isolates were identified to the species level using the following biochemical tests: triple sugar iron, urea, oxidase, motility, indole, and ornithine decarboxylase and by the API 20 E identification system (bioMérieux Inc.).

Determination of Clinical and Subclinical Mastitis

Clinical mastitis was defined as the presence of abnormal milk, and/or abnormal udder, and/or systemic signs of IMI that warranted intramammary and/or anti-inflammatory therapies. Milking personnel identified cows with clinical mastitis, and treatment was

administered following the collection of samples from the infected quarters. Subclinical mastitis was defined as the presence of the same pathogen in the same quarter in at least 2 of 3 consecutive samples. Data for each cow were combined over time points and quarters to obtain percentages of subclinical and clinical mastitis cases. Final analyses were conducted using the percentages on a per-cow basis. For example, at one collection time point, a cow has 4 data points, one for each quarter. A cow with 4 lactations with 5 collection time points in each lactation had 80 total observations ($4\text{ quarters} \times 5\text{ collection time points} \times 4\text{ lactations} = 80\text{ total observations}$). If this cow had 2 subclinical events, she would have a 2.5% percentage. The same would hold true if she experienced 2 clinical events.

Subclinical mastitis caused by *Corynebacterium bovis*, coagulase-negative staphylococci, and *Staphylococcus aureus* were observed in the Holstein herd, but the majority of clinical mastitis was due to *Staph. aureus* and *Streptococcus uberis*. The majority of subclinical mastitis in the Jersey herd was due to coagulase-negative staphylococci, *Strep. uberis*, and *Staph. aureus*; *Strep. uberis* and *Escherichia coli* caused the majority of clinical mastitis.

Determination of SCS and Milk Production Data

The number of somatic cells in milk was determined by Dairy Herd Improvement Association personnel at The University of Tennessee, Knoxville with a Soma-count 300 cell counter (Bentley Instruments, Chaska, MN). Somatic cell scores and projected 305-d mature equivalent milk yields were obtained from Dairy Records Management Systems (Raleigh, NC).

Statistics

All statistical calculations were carried out with SAS version 9.0 (SAS Institute, Cary, NC). A randomized block design blocking on lactation with covariates on SCS and milk yield was used to determine effects of CXCR2 SNP and haplotype genotypes on the percentage incidence of subclinical and clinical mastitis over lifetime for each breed. Analysis of variance was used to detect significance differences between data. Data are presented as least squares means with associated standard error. Randomized block designs blocking on lactation were used to determine effects of CXCR2 SNP on SCS and milk yield. Correlations between percentage of subclinical and/or clinical mastitis incidence, SCS, and milk yield were determined. Statistical significance was declared at $P < 0.05$.

Figure 1. CXCR2 single nucleotide polymorphism (SNP) +777 genotype by estimated observations positive for subclinical mastitis (%). Data are means $\pm$ SEM. Letters in caps represent LSM separation for Holstein cattle ($P < 0.05$). n = number of cows expressing CXCR2 SNP +777 genotype.

RESULTS

Analysis of SNP +777 Genotypes with Subclinical and Clinical Mastitis

Holstein cows exhibited higher estimated means of percentage subclinical mastitis for all SNP when compared with Jersey cows. Single nucleotide polymorphisms +612, +777, and +861 showed a significant association with subclinical mastitis ($P < 0.05$). Further attention focused on SNP +777, as SNP +612 and +861 are synonymous polymorphisms. SNP +777 (G to C) results in a Gln²⁴⁵ to His²⁴⁵ replacement, which may affect mastitis phenotype. Cows with genotype CC at SNP +777 expressed increased percentages of subclinical mastitis when compared with cows with genotype GG ($P < 0.05$): 37% vs. 22%, respectively (Figure 1). This difference was not observed in Jerseys (Figure 1) ($P > 0.10$). Significant differences were not detected in either breed between SNP +777 genotypes and percentage observations positive for clinical mastitis (Figure 2). However, Holstein cows tended to have lower incidence of mastitis when the CC genotype was expressed compared with the GG phenotype.

Association of SNP +777 Genotypes with SCS and Milk Yield

The relationship between SNP +777 genotypes and SCS was evaluated. Single nucleotide polymorphism

Figure 2. CXCR2 single nucleotide polymorphism (SNP) +777 genotype by estimated observations positive for clinical mastitis (%). Data are means $\pm$ SEM. n = number of cows expressing SNP +777 genotype.

+777 had a significant association with SCS in Holsteins ($P < 0.001$); however, this polymorphism was not significant for Jersey SCS differences (Table 2). Cows expressing genotypes GG and GC had increased SCS relative to the CC genotype; however, only cows expressing the GC genotype had significantly greater SCS compared with those expressing genotype CC. No significant differences were observed for Jersey SNP +777 genotypes and SCS, which ranged from 3.09 to 3.40.

We observed no significant differences between SNP +777 genotype and 305-d milk yield in Jerseys, as estimated means ranged from 8419 to 8629 kg (Table 2). However, Holsteins expressing genotype GG had significantly lower 305-d milk yields (9412 kg) vs. Holsteins expressing genotypes GC and CC (10,231 and 10,056 kg), respectively.

Table 2. Bovine CXCR2 single nucleotide polymorphism (SNP) +777 genotype by SCS and milk yield over all lactations.

Holstein SNP +777 genotype	Estimated SCS	Estimated 305-d milk yield (kg)
GG	3.10 (± 0.15) ^a	9412 (± 151) ^b
GC	4.00 (± 0.11) ^b	10,231 (± 112) ^a
CC	2.72 (± 0.20) ^a	10,056 (± 220) ^a
Jersey SNP +777 genotype		
GG	3.09 (± 0.07)	8419 (± 53)
GC	3.35 (± 0.13)	8585 (± 105)
CC	3.40 (± 0.40)	8629 (± 326)

^{a,b}Least square mean separation within herd and column ($P < 0.05$).

Table 3. Correlation coefficients among Holstein SCS, milk yield, clinical and subclinical mastitis, and lactation.

	Correlation coefficient			
	Percentage of subclinical mastitis	Milk yield	SCS	Lactation
Percentage of clinical mastitis	-0.04	0.12 ^a	0.11 ^a	0.19 ^a
Percentage of subclinical mastitis		0.06	0.02	0.15 ^a
Milk yield			-0.06	0.38 ^a
SCS				0.17 ^a

^a $P < 0.05$.

Correlation Coefficients

Weak positive correlations were observed between percentage of clinical mastitis and SCS (0.11) and projected 305-d milk yield (0.12) in Holsteins (Table 3). In contrast, little if any correlation was observed between subclinical mastitis and SCS or milk yield. As expected, lactation number was correlated with mastitis incidence, SCS, and milk yield.

Percentage of subclinical mastitis and SCS were moderately correlated (0.40) in Jerseys (Table 4). Clinical mastitis incidence also was weakly correlated with SCS (0.20). In contrast to the Holstein population, a moderate negative correlation was observed in Jerseys for SCS and milk yield (-0.28). Similar to the Holstein population, a weak positive correlation was observed between lactation and percentage of subclinical mastitis (0.21) and SCS (0.20). However, a weak negative correlation (-0.11) was observed between lactation and milk yield.

DISCUSSION

We previously identified 10 CXCR2 haplotypes created by 5 SNP located within the predicted coding

Table 4. Correlation coefficients among Jersey SCS, milk yield, clinical and subclinical mastitis, and lactation.

	Correlation coefficient			
	Percentage of subclinical mastitis	Milk yield	SCS	Lactation
Percentage of clinical mastitis	0.06	0.05	0.20 ^a	0.00
Percentage of subclinical mastitis		-0.01	0.40 ^a	0.21 ^a
Milk yield			-0.28 ^a	-0.11 ^a
SCS				0.20 ^a

^a $P < 0.05$.

region of the bovine CXCR2 gene (Youngerman et al., unpublished, 2003). CXCR2 SNP +612, +777, and +861 were significantly ($P < 0.05$) associated with incidence of subclinical mastitis. We focused our attention on SNP +777, as it resulted in a change at amino acid 245 (Gln to His). As such, this nonsynonymous polymorphism may be subject to natural selection, because it may ultimately affect mastitis phenotype (Hansson and Westerberg, 2002; Cardon and Palmer, 2003). Because of the tight linkage of these SNP, as well as the fact that SNP +612, +777, and +861 have near identical allele and genotype frequencies within the Holstein breed, it was not unusual to expect that synonymous SNP +612 and +861 showed similar results as nonsynonymous SNP +777 (Youngerman et al., unpublished, 2003).

Because of potential effects caused by the nonsynonymous SNP +777 (G to C) on CXCR2 signal transduction and function, we examined the association of SNP +777 genotypes with percentages of subclinical and clinical mastitis incidence. A nonsignificant decrease of clinical mastitis incidence was observed in cows that expressed genotype CC. A significant difference ($P < 0.05$) between subclinical mastitis incidence and SNP +777 genotype was identified also. Holsteins expressing the GG genotype had a lower incidence of subclinical mastitis than cows that expressed the CC genotype. Interestingly, greater 305-d milk yield was observed in Holsteins that expressed genotype CC when compared with those expressing GG and would support prior research that concluded cows selected for greater milk yields also have increased mastitis infections (Shook, 1993; Hansen, 2001). More surprising was the lack of significant SCS differences between SNP +777 genotypes GG or CC, as we expected cows with a CC genotype to have greater concentrations of somatic cells because of more bacterial infections. However, bacterial presence does not always increase SCS. The correlation between SCS and clinical mastitis has typically ranged from 0.6 to 0.8, suggesting that the relationship between SCS and clinical mastitis is not absolute (Emanuelson et al., 1998). The absence of increased SCS despite bacterial presence suggests a potential defect in one or more of the mechanisms necessary for neutrophil migration to the mammary gland, including bacterial recognition, cell-to-cell communication, and/or intracellular communication.

The amino acid change resulting from the G to C substitution at SNP +777 offers a possible explanation for increased bacterial presence in Holstein cattle expressing the CC genotype. During the onset of mastitis, different pathogens invade the mammary gland and elicit cytokine and chemokine release from macrophages and surrounding epithelial cells (Paape et al.,

2002). Inflammatory mediators, such as interleukin-8, growth-related oncogene- α , and neutrophil-activating peptide-2, bind to CXCR2 receptors and induce CXCR2 internalization. The subsequent cascade of signals results in the release of intracellular Ca^{2+} and granular enzymes, as well as initiates chemotaxis (Loetcher et al., 1994; Ahuja et al., 1996; Knall et al., 1996). Although studies have identified binding sites in the N' terminus that are responsible for binding selectivity and in the C' terminus for receptor phosphorylation, few have focused on identifying functional roles of intracellular loops (Ben-Baruch et al., 1995; Ahuja et al., 1996; Richardson et al., 2003). Damaj et al. (1996) determined that Met²⁴¹ is important in G-protein binding and intracellular Ca^{2+} mobilization. Damaj et al. (1996) also determined that other amino acids in the third intracellular loop were moderately involved in mediating intracellular signaling. As the polymorphism at +777 results in an amino acid change at position 245 in the third intracellular loop, it has the potential to interfere with G protein-mediated signaling and subsequent neutrophil functions, such as migration. This hypothesis is supported by the lack of increased SCS in cows expressing the CC genotype. Research is currently underway to determine the functional activities of neutrophils collected from cows expressing genotype GG compared with CC at SNP +777.

Another potential explanation for increased subclinical mastitis in Holstein cattle with the CC genotype at SNP +777 may be due to expression of other genes located near CXCR2 on BTA 2. The natural resistance associated macrophage protein-1 is a potential gene affecting mastitis and related immune responses as it is located near CXCR2 (1.3 cM) and has been shown to be polymorphic and associated with immune function (Blackwell, 1989; Bellamy, 1999). Single nucleotide polymorphisms located within other regions of the bovine CXCR2 gene may also affect CXCR2 function during mastitis infections. Several single nucleotide polymorphisms have been detected within different regions of the human CXCR2 gene (Kato et al., 2000; Renzoni et al., 2000). As such, other nonsynonymous SNP located within segments of the CXCR2 coding sequence may affect receptor binding and function. Additionally, either synonymous or nonsynonymous SNP in the promoter and 3' untranslated regions may affect initiation and/or termination of transcription or translation. To determine whether other SNP are involved with changes in susceptibility or resistance, the complete sequence of the bovine CXCR2 gene should be determined.

These results are extremely promising; however, it is noted that these results are based upon a relatively small cattle population and need to be validated. In

addition, the small sample size precluded the ability to evaluate effectively the association of mastitis with specific SNP haplotype genotypes that would more specifically identify an animal's genotype. Thus, current research efforts are aimed at expanding the study presented here.

In conclusion, we identified 3 polymorphisms within the CXCR2 gene that were associated with subclinical mastitis. This research is promising, as it may represent an effective means of marker-assisted selection for mastitis resistance and potentially other inflammatory diseases. Moreover, the ability to genetically identify mastitis susceptible vs. resistant animals gives researchers an opportunity to determine what mechanisms are linked to disease susceptibility and may open the door to novel preventive and therapeutic measures for mastitis.

ACKNOWLEDGMENTS

The authors express their gratitude for the dedication of Barbara Gillespie, Susan Headrick, and Mark Lewis within the Mastitis Research Lab for collecting and processing thousands of milk samples from these herds each year. The aid of Henry Dowlen, Hugh Moorehead, and Phillip Lunn at the Lewisburg Dairy Experiment Station, as well as Dennis Onks, David Johnson, and Ken Lamar at the Spring Hill Experiment Station for their help in collecting samples also is appreciated greatly. The research reported within this manuscript was supported by The University of Tennessee Food Safety Center of Excellence and The University of Tennessee Agricultural Experiment Station.

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