Chapter 4: Associations between Specific Bovine Leukocyte Antigen DRB3 alleles and Mastitis in Canadian Holsteins

4.1 Introduction

4.1.1 MHC and BoLA Information

The major histocompatibility complex (MHC) is a large cluster of tightly linked genes that play a well-documented role in the immune system (Klein 1986, Andersson & Davies 1994). The products of these genes are involved in the induction and regulation of immune response. The MHC spans approximately 4 Mb of the human genome, 1.5 Mb in mice and approximately 2.5 Mb of the cattle genome (Lewin 1996; Rothschild et al. 2000). In humans, the MHC is located on chromosome 6 whereas in cattle, it is located on chromosome 23. It has been estimated that the mammalian MHC contains over 200 genes (The MHC sequencing consortium 1999). The structure and organization of the MHC genes of cattle, known as the bovine leukocyte antigen (BoLA) complex, are very similar to those of the human MHC. The genes are organized into three distinct classes (class I, II, and III). Each of these classes is divided into regions and sub-regions, containing genes and pseudogenes. The major difference between the organization of the BoLA complex with that of the human MHC is the BoLA complex is found in two separate regions of the chromosome rather than a single cluster of genes seen in most mammals (Andersson et al. 1988; van Eijk et al. 1993). The larger gene cluster is located at BTA23 band 22 and apparently contains all of the bovine class I and class III sequences, and genes encoding both subunits of the classical class II proteins DQ and DR. The remaining BoLA class II loci (DIB, DNA, DOB, DYB, TCP1, LMP2, LMP7 and TAP2) are located in a cluster near the centromere at BTA23 band 12-13.
(Rothschild et al. 2000). Comparative analysis suggests that the disruption was likely caused by a single chromosomal inversion (Band et al. 1998).

The class I and class II genes encode primarily for cell surface glycoproteins involved in antigen presentation and immune regulation. The class I glycoproteins are present on most nucleated cell types and are involved in the MHC restriction of CD8+ cytotoxic T-cell (T<sub>C</sub>) activation and antigen recognition. The class II glycoproteins are mainly found on the surface of professional antigen presenting cells and are involved in the MHC restriction of CD4+ T-helper cell (T<sub>H</sub>) activation and antigen recognition. The class III genes encode secreted proteins, some of which are associated with the immune regulation, including soluble serum proteins, several components of complement system and tumor necrosis factors (alpha and beta). Of the class II genes, cattle express one DR gene pair (DRA and DRB3) and one or two DQ gene pairs per haplotype. The coding sequence of DRA is monomorphic, while the DRB3 gene has over 103 identified alleles (Takeshima et al. 2002). As a result of this polymorphism as well as its functional importance, the DRB3 locus and its gene products are among the best defined in cattle.

As MHC molecules function to present peptide sequences to T-cells, the particular set of MHC molecules expressed by an individual influence the repertoire of antigens to which individual T<sub>H</sub> and T<sub>C</sub> cells can respond. MHC class I molecules are able to present very different antigenic peptides than MHC class II molecules. The MHC class I molecules generally present short peptides (nine amino acids) with anchor residues that assist in binding the peptide to the MHC. On the other hand, MHC class II molecules are able to present much larger peptides (13-18 amino acids) (Engelhard 1994). For these reasons, the MHC partly determines the response of an individual to
antigens of infectious organisms. Consequently, the MHC has, as is logical, been implicated in susceptibility and resistance to infectious disease (Gavora & Spencer 1983; Hill 1998, 2001).

4.1.2 Associations between MHC and Disease

As a result of their role in antigen presentation, MHC alleles have been examined for associations with various autoimmune and infectious diseases. Many MHC-disease associations have been found in mice, chickens and humans. The role between the MHC and disease susceptibility in mice was demonstrated by Lilly et al. (1964) using two MHC-distinct strains of mice, C3H and C57-BL6 that were inoculated with Gross leukemia virus. The C3H mice were much more susceptible to the virus. The situation was reversed, however, in the case of infection with Schistosoma mansoni where the C57-Black were more susceptible. The two strains of mice differed only in the H-2 (MHC) region.

The most widely cited example of an MHC-disease association because of its commercial application, is that of Marek’s disease in chickens. The B21 haplotype has been shown to be associated with resistance Marek’s disease (Briles et al. 1977). In humans, many associations have been demonstrated, including susceptibility to ankylosing spondylitis (AS), narcolepsy, typhoid and yellow fever, leprosy, as well as protection from severe malaria, persistent hepatitis-B virus (HBV) infection and HIV infection, among many others (Giphart et al. 1990; De Vries et al. 1989; Ottenhoff et al. 1986; Hill et al. 1991; Thursz et al. 1995). The information provided by these associations is of great importance as it may be used in the development of new vaccine strategies for protection against these diseases.
4.1.3 Associations between BoLA and Disease

Associations between BoLA alleles and disease have also been identified in cattle (class I and class II genes). Class I associations include tick resistance (Stear et al. 1984, 1989b), nematode egg worm counts (Stear et al. 1989a, 1990), resistance to persistent lymphocytosis caused by bovine leukemia virus (BLV) (Stear et al. 1988), chronic posterior spinal paresis (PSP) (Park et al. 1993) ketosis (Mejdell et al. 1994), resistance to dermatophilosis (Maillard et al. 1996) as well as mastitis (Solbu 1982; Oddgeirsson et al. 1988; Solbu & Lie 1990; Mejdell et al. 1994; Schukken et al. 1994).

In humans, the number and the significance of the MHC-disease associations increased dramatically following the development of class II typing techniques (Giphart et al. 1990). The same has been seen in cattle, as a result of the PCR-RFLP class II typing technique developed by van Eijk et al. (1992). Associations have been demonstrated between genes in this region and diseases such as decreased risk of cystic ovarian disease and retained placenta (Sharif et al. 1998), resistance to persistent lymphocytosis caused by BLV (Xu et al. 1993) and mastitis (Lundén et al. 1990; Schukken et al. 1994; Sharif et al. 1998).

4.1.4 Associations between BoLA-DRB3 Alleles and Mastitis and Somatic Cell Score

Of the class II genes, the DR locus has been the most widely studied of the BoLA complex genes because it is extremely polymorphic, with over 103 different alleles identified (Takeshima et al. 2002). Some of these alleles have been shown to be
associated with disease traits. Of particular interest, due to the prevalence and high
disease costs, are associations between the DRB3 alleles and mastitis.

Dietz et al. (1997) demonstrated that DRB3.2*16 (DRB3*1501-1502) was a risk
factor for high acute SCC. Kelm et al. (1997) showed a similar association between
allele *16 and increased somatic cell score (SCS). In contrast, however, Starkenburg et
al. (1997) demonstrated that a decreased SCS for cows in 2\textsuperscript{nd} lactation was associated
with the DRB3.2*16 allele. Sharif et al. (1998) also found a significant decrease in SCS
associated with allele DRB3.2*16. The studies by Dietz et al., Starkenburg et al. and
Kelm et al., were performed on relatively small groups of US Holstein (n=584, 173 and
137, respectively). The findings of Sharif et al. (1998) were from a larger population
consisting of 835 Canadian Holsteins. The size of the population studied, experimental
design, allele frequencies or different states of linkage disequilibrium may have
contributed to the discrepancies between these results.

Other alleles have also been shown to influence SCC. Dietz et al. (1997) showed
that allele DRB3.2*22 (DRB3*1101) was associated with a decrease in SCC, while
DRB3.2*23 (DRB3*2701-2703, 2705-2707) was associated with an increase.

Various alleles have been implicated in susceptibility and resistance to clinical
mastitis. Allele DRB3.2*8 (DRB3*1201) has been shown to be associated with
susceptibility to clinical mastitis, while alleles DRB3.2*11 (DRB3*0901-0902, 1202) and
DRB3.2*23 were associated with increased resistance to clinical mastitis (Kelm et al.
1997). Sharif et al. (1998) demonstrated a significant association between clinical
mastitis and allele DRB3.2*23.
4.1.5 Experimental Objectives

The findings of Kelm et al. (1997), Dietz et al. (1997) and Sharif et al. (1998), strongly suggest that associations exist between alleles DRB3.2*16 and DRB3.2*23 with mastitis and SCS in dairy cattle. However, the lack of consensus among these results indicates a need for further investigation to determine the precise nature of these associations; for example, a study involving of a larger population, for which extensive health records are available. Mastitis is a complex disease caused by multiple pathogens and may be present at both a clinical or subclinical level. For this study, clinical mastitis, subclinical mastitis, mastitis at calving as well as SCS were examined. The objective was to use the MPT-PCR technique described by Ledwidge et al. (2001) to rapidly genotype a large population of cows for alleles DRB3.2*16 and *23 only in order to investigate associations between these alleles and mastitis incidence in Canadian Holsteins.

4.2 Materials and Methods

4.2.1 Animal Information and Data Collection

4.2.1.1 Sentinel Herd Information

The Sentinel Herd Project was developed through a partnership among the University of Guelph, the Ontario Ministry of Agriculture and Foods, Ontario Dairy Herd Improvement, the Dairy Farmers of Ontario and the Ontario Association of Bovine Practitioners (Kelton et al. 1999). Forty Ontario veterinary practitioners were involved in the project, and a total of 60 of their dairy producer clients were selected to participate. The study took place over an 18-month period between July 1997 and December 1998. Fifty-eight of the herds involved in the project were Holstein herds; the other two were
Jersey herds. The herds are located in 23 counties across Ontario and are typical of the demographic range in the Ontario dairy industry (Kelton et al. 1999). The herd size ranged from 25 to 170 milking cows with a mean production range between 150 and 250 milk breed class average (BCA) and a bulk tank SCC range from less than 100 000 to over 400 000 cells/ml. The majority of the herds (47) were housed in tie-stall barns, with the remaining in free stalls (11) and bedded pack facilities (2).

From each herd, detailed production and health data were collected. This data included multiple composite milk samples, blood samples as well as several management questionnaires. The enrollment and follow-up questionnaires focused mainly on animal housing, feeding management, milking systems, milking management, mastitis diagnosis, mastitis treatment, overall health management and biosecurity practices. The third questionnaire focused on calf and heifer management during the 18-month period.

At scheduled intervals throughout the study as described below, milk samples were collected from all four quarters and a composite sample was cultured. Milk samples were initially frozen, and later thawed for culturing. The interpretation of the culture results was based on the guidelines of the National Mastitis Council (NMC 1996). The three most predominant cultured organisms were recorded along with a score, for each organism, from 0-4 based on the number of colonies. The organism names were recorded if they were known mastitis-causing pathogens. Otherwise, the cultures were recorded as NBP (no bacterial pathogen) or NBG (no bacterial growth).

Initial composite milk samples were taken from all lactating cows in July and August of 1997. Subsequent collections were carried out every four months until the end of the study in December 1998. For the sake of clarity, the milk culture results from the
scheduled herd visits shall be known as herd cultures. The total number of herd cultures collected from animals with accompanying whole blood samples was 11683. Composite milk samples were also collected from 70% of all fresh cows within 48 hours of calving. The total number of milk samples collected at calving from animals with blood samples was 2391. Finally, clinical mastitis cases, detected by the herd owner, were recorded and quarter samples were collected and cultured. The total number of clinical mastitis cases recorded on animals with accompanying blood samples was 1070. Clinical case detection was dependent upon the producer’s ability to recognize the occurrence of mastitis.

Whole blood samples were collected from 3493 cows in 58 herds during the summer of 1998. Samples were collected from all cows in milk at the time of collection. In some herds, samples were also collected from dry cows.

Data were also collected on herd production and milk quality. Through the DHI DairyComp 305™ database, production and udder health data were also available for individual cows. Appendix 1 lists all information made available through the Sentinel Herd Project.

4.2.1.2 Somatic Cell Score and Production Data

Cow EBVs for SCS and production traits were obtained from the national genetic evaluations of May 2001, provided by the Canadian Dairy Network (CDN). All fully registered cows, whose records had passed CDN edits for use in genetic evaluations, were included in the analysis. The EBVs were used, instead of the phenotypic SCS information available through the Sentinel Herd Project, because the EBVs are corrected for systematic fixed effects such as herd, age and season, as well as random effects.
(Schaeffer et al. 2000). As a result, the EBVs can be used to directly estimate the genotype effect. Appendix 2 lists the SCS and production data obtained from CDN.

4.2.2 Sample Preparation

4.2.2.1 Blood Collection and White Blood Cell Preparation

Blood collection and preparation of white blood cells (WBC) was completed between June and August 1998 from 58 of the 60 herds participating in the study. Blood was not collected from two of the herds; in one case, the herd was sold prior to the summer of 1998, and in the other case, the owner was unable to schedule a suitable time for collection. The blood samples from the two Jersey herds were excluded from this study, leaving 56 Holstein herds. The WBC were extracted from the fresh whole blood using the following procedure: The blood collection vacuum tubes were balanced and centrifuged at room temperature for 30 minutes at 200g. Approximately 2-3 ml of the WBC were harvested using a transfer pipette and placed in a 15 ml plastic conical tube. Double distilled, or milliQ™, water was added to a total volume of 6 ml followed by 6 ml of phosphate buffered saline (PBS). The tubes were then sealed, inverted 3 times, balanced and centrifuged at 200g for 15 minutes. After ensuring that a pellet had formed in the bottom of the tubes, the supernatant was decanted and the pellet re-suspended in 6 ml of PBS. The tubes were once again inverted 3 times, balanced and centrifuged at 200g for 15 minutes. The supernatant was again decanted and the pellet re-suspended in 3 ml of PBS. One ml of the suspension was then aliquotted into each of 3 separate cryovial tubes and samples were then frozen at -70°C until time of DNA extraction.
4.2.2.2 DNA Extraction

DNA extraction from the frozen WBC lysates was performed as described by Ledwidge et al. (2001). Briefly, the WBC lysates were thawed and subjected to enzymatic digestion with proteinase K (Sigma-Aldrich Canada, Oakville, ON, Canada). The lysate, along with proteinase K buffer and proteinase K, was incubated at 56°C for 3 hours. The enzyme was then inactivated by incubation at 95°C for 10 minutes.

4.2.3 Genotyping by Multi-Primer Target-Polymerase Chain Reaction

Genotyping was performed as described by Ledwidge et al. (2001). Briefly, the DNA was amplified by PCR using four primers. Two outer primers amplified the entire gene fragment while two inner primers simultaneously amplified the DRB3.2*16 and DRB3.2*23 alleles. The resulting PCR fragments were then separated using 1.5% agarose gel electrophoresis. Of the 3157 blood samples collected, 2200 were successfully genotyped for the presence of DRB3.2*16 and *23. DRB3.2*16 was present in 434 samples, DRB3.2*23 in 155, both alleles were present in 30, and 1581 samples contained neither allele.

4.2.4 Data Editing and Trait Definitions

4.2.4.1 Pedigree Information

Pedigree information for each cow was obtained from CDN. A list of 3493 cows from the Sentinel Herd with DNA samples was edited to remove cows with unknown and non-Holstein registration numbers, leaving a total of 3157 cows. A pedigree extraction routine was used to extract pedigree records of all known ancestors from the CDN
database in May 2001, yielding 8449 records. This file was reduced to two generations of pedigree information (6017 records). Using this information and edited data files specific to the analyses outlined in sections 2.4.1-2.4.3 below, ordered pedigree files were constructed.

4.2.4.2 Lactation Number

Cows were divided into three categories (1\textsuperscript{st}, 2\textsuperscript{nd}, and 3\textsuperscript{rd} lactation or greater) based on their lactation number at the beginning of the 18 month study period.

4.2.4.3 Clinical Mastitis

Clinical mastitis cases were identified by the producers and, as a result, were dependant upon the producer’s ability to detect the occurrence of mastitis. As a general rule, a clinical case was assumed if there was inflammation of the udder and/or abnormal milk.

All cows with an identified case of clinical mastitis were treated as positive, regardless of whether a bacterial pathogen was isolated from the sample. The number of clinical cases during the trial was tallied for each cow.

Variance Component Estimation (VCE) analysis was used to estimate the heritability ($h^2$) of number of clinical mastitis cases (VCE 4.1, Neumaier & Groenveld 1998). The data sets used for both the VCE and mixed model analysis included all animals successfully genotyped by the MPT-PCR method.

4.2.4.4 Subclinical Mastitis

Bacterial cultures obtained from composite milk samples, from all cows milking at the scheduled herd visits, were used as an indicator of subclinical mastitis. A positive
culture without the presence of abnormal milk or inflammation suggested the presence of a subclinical infection. Herds were tested between 4 and 7 times, with the majority visited 5 times within the 18 months of the trial. For each culture, the three most predominant organisms cultured were recorded and a final bacteriological interpretation was made. The final bacteriological interpretation consisted of the name of the predominant organism cultured. If no bacterial pathogen or no bacterial growth was detected, the interpretation was recorded as NBP or NBG, respectively.

For each cow, two calculations were made from the final bacterial interpretations: 1) the number of cultures positive for bacteria, and 2) the number of cultures positive for a mastitis-causing bacterial pathogen (Table 4.1).

VCE analysis was again used to estimate the $h^2$ of subclinical mastitis. The $h^2$ were estimated for both the presence of bacteria in the herd cultures as well as the presence of a bacterial pathogen in the cultures. The data sets used for the analysis included all genotyped animals with at least one herd culture.

4.2.4.5 Mastitis at Calving

Positive cultures for bacteria or bacterial pathogens, in milk samples collected within 48 hours of calving, were determined in a similar manner to that for subclinical mastitis. Cows were scored as positive for a bacterial pathogen if they cultured positive for a mastitis-causing pathogen, and were scored positive for bacteria if they cultured positive for any type of bacteria (not necessarily a pathogen).

Several cows calved twice during the trial period. In these cases, only the composite sample taken following the first calving was used in the statistical analysis.
All genotyped cows with at least one sample at freshening were included in the mixed-model analysis.

### 4.2.5 Statistical Analysis

Initial analyses were performed using the software VCE 4.1 (Neumaier & Groenveld 1998). The Restricted Maximum Likelihood (REML) method of VCE was used to estimate the $h^2$ of number of cases of clinical mastitis, the number of cases with presence of bacteria or absence and pathogens in herd cultures, as well as the presence of bacteria and pathogens at calving. Subsequent analyses, including the final estimation of effects of BoLA genotype and contrasts, were performed using SAS PROC MIXED (SAS Institute Inc., 1999) as outlined below.

#### 4.2.5.1 Models

##### 4.2.5.1.1 Clinical Mastitis

The following mixed model was analyzed using SAS PROC MIXED (SAS Institute Inc., 1999) and 6017 known ancestors were included in the pedigree in order to build the relationship matrix among the 2200 cows with records. Animal was a random effect, included to account for polygenic background variation. All other independent variables were fixed effects. BoLA genotype, herd and lactation were classification variables. To determine the factors that influenced the number of clinical mastitis cases, the following model was used:

$$Y_{ijkl} = G_i + H_j + L_k + A_l + \varepsilon_{ijkl}$$  \[\text{Model 1}\]

where $Y_{ijkl} =$ number of clinical cases, $G_i =$ fixed effect of the $j^{th}$ BoLA-DRB3.2 genotype ($j= -/-, -/16, 16/23, -/23$), $H_j =$ fixed effect of the $k^{th}$ herd ($k=1-56$),
L_k = fixed effect of the l^{th} lactation (l=1, 2, 3+),
A_i = random effect of animal i (i=1-2200), and
\varepsilon_{ijkl} = residual error.

4.2.5.1.2 Subclinical Mastitis

To determine the factors that affected the presence of bacteria in herd cultures, the following model was used:

\[ Y_{ijkl} = \beta X_i + G_j + H_k + L_l + A_i + \varepsilon_{ijkl} \]  
[Model 2]

where

- \( Y_{ijkl} \) = number of cultures positive for bacteria,
- \( X_i \) = total number of cultures for animal i (i=1-2118),
- \( \beta \) = regression of number of positive cultures on total number of cultures,
- \( G_j \) = fixed effect of the j^{th} BoLA-DRB3.2 genotype (j= -/-, -/16, 16/23, -/23),
- \( H_k \) = fixed effect of the k^{th} herd (k=1-56),
- \( L_l \) = fixed effect of the l^{th} lactation (l=1, 2, 3+),
- \( A_i \) = random effect of animal i (i=1-2118), and
- \( \varepsilon_{ijkl} \) = residual error.

To determine the factors that affected the occurrence of bacterial pathogens in herd cultures, the model used was identical to Model 2, with the exception that \( Y_{ijkl} \) represents the number of cultures positive for bacterial pathogens. Animal was a random effect; all other independent variables were fixed effects. Genotype, herd and lactation were classification variables.

This same model was also used to investigate associations between BoLA genotype and number of herd cultures positive for a specific pathogen. In each case, \( Y_{ijkl} \) represents the number of positive cultures for one of the three most common pathogens, \( Escherichia coli \) (n=106), \( Stretococcus \) spp. (n=344) and \( Staphylococcus aureus \) (n=631).

Note that 82 cows were lost in this analysis because there was no herd visit during the period that they were present in the herd.
4.2.5.1.3 Mastitis at Calving

A total of 2143 samples were collected from 1306 cows. Only the first calving for each cow was used in the analysis.

To determine the factors that affected the presence of bacteria at calving, the following model was used:

\[ Y_{ijkl} = G_i + H_j + L_k + A_l + \varepsilon_{ijkl} \]  \[\text{[Model 3]}\]

where \( Y_{ijkl} \) = number of cultures positive for a bacteria (0 or 1),
\( G_i \) = fixed effect of the \( i^{\text{th}} \) BoLA genotype (\( i = -/-, -/16, 16/23, -/23 \)),
\( H_j \) = fixed effect of the \( j^{\text{th}} \) herd (\( j = 1-55 \)),
\( L_k \) = fixed effect of the \( k^{\text{th}} \) lactation (\( k = 1, 2, 3+ \)),
\( A_l \) = random effect of animal \( l (l=1-1306) \), and
\( \varepsilon_{ijkl} \) = residual error.

To determine the factors that affected the presence of bacterial pathogens at calving, the model used was identical to Model 3, with the exception that \( Y_{ijkl} \) represents a culture positive for a bacterial pathogens. Animal was a random effect, all other independent variables were fixed effects. Genotype, herd and lactation were classification variables.

4.2.5.1.4 Somatic Cell Score and Production Traits

The model for analysis of SCS and production EBVs was:

\[ Y_i = G_i + \varepsilon_i \]  \[\text{[Model 4]}\]

where \( Y_i \) = the dependent variables,
\( G_i \) = fixed effect of the \( i^{\text{th}} BoLA-DRB3.2 \) genotype (\( i = -/-, -/16, 16/23, -/23 \)), and
\( \varepsilon_i \) = residual error.
BoLA genotype was a classification variable. The dependent variables were EBV milk, EBV milk 1st lactation, EBV fat, EBV fat 1st lactation, EBV fat percent, EBV protein, EBV protein 1st lactation, EBV protein percent, test day (TD) protein 1st lactation, estimated transmitting ability (ETA) SCS, ETA SCS 1st lactation, ETA persistency, ETA persistency 1st lactation. The models were analyzed using SAS PROC GLM (SAS Institute Inc., 1999).

4.2.5.2 Contrasts

Three non-orthogonal statistical contrasts were made for each model based on the results of previous studies (Kelm et al. 1997; Starkenburg et al. 1997; Dietz et al. 1997; Sharif et al. 1998). These studies have demonstrated associations between BoLA-DRB3.2*16 and SCS, as well as associations between BoLA-DRB3.2*23 and incidence of clinical mastitis. The objective here was to determine whether similar associations could be confirmed in the Sentinel Herd population. As a result, the following contrasts were used in the analyses:

1) Genotype DRB3.2*16 versus all other genotypes; 2) Genotype DRB3.2*23 versus all other genotypes; and 3) Genotype DRB3.2*16/*23 versus all other genotypes.

Even though only 20 animals were heterozygous for alleles DRB3.2*16 and *23, it was of interest to investigate whether the effect of one allele was stronger than the other as the results of previous studies indicate that *16 and *23 contribute to the resistance and susceptibility to mastitis, respectively (Sharif et al. 1998). At present, there is no research to indicate the effect of the presence of both alleles simultaneously on susceptibility to the disease.
4.3 Results

4.3.1 Clinical Mastitis

In total, 1042 cases of clinical mastitis were diagnosed in 657 cows. Milk samples were collected and cultured from the affected quarter. Of these cultures, 539 showed no growth of a bacterial pathogen and 28 were contaminated. Of the remaining 475 cultures, 78 had growth of a contagious pathogen and 397 had growth of an environmental pathogen. The most commonly isolated bacteria were *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus dysgalactia* with 61, 156 and 160 positive cultures, respectively (Table 4.2).

Using VCE analysis the $h^2$ calculated for clinical mastitis was 0.09. Given the limited data available for estimation of the $h^2$ and the consequent lack of precision, mixed models to estimate BoLA allele effects were fit for different levels of $h^2$ to test the sensitivity of allele effects to this parameter. The results for clinical mastitis at $h^2=0$, 0.05 and 0.10 are given in Table 4.3. An examination of the results at $h^2=0$, 0.05 and 0.10, shows little variation between the three levels, indicating that the polygenic background information contributes little to the model. For this reason, only the results for $h^2=0$ will be discussed.

Cows were present in the herds for varying periods of time. The time during which a cow was capable of contracting mastitis was different for each individual. For this reason, a “period-at-risk” was calculated for each cow. The risk period included the time from which the trial began or the animal calved (whichever occurred last) to the time that the animal was culled or the trial ended (whichever occurred first). It was initially believed that the number of clinical cases might be related to the period-at-risk.
For example, animals with a high number of clinical cases in a short time period might be removed from the herd. On the other hand, animals that were culled early for various reasons might not have the opportunity to develop multiple cases of clinical mastitis. An examination of the relationship between number of cases and period-at-risk showed no associations between the two traits. As a result, period-at-risk was not included in the model as a covariate.

For clinical mastitis, the fixed effects of herd and lactation were significant (p<0.05). Genotype had no overall significant effect on the number of cases of clinical mastitis. Allele $DRB3.2*16$ was contrasted against all others for both traits. The same contrasts were performed for allele $DRB3.2*23$. No associations were found between the alleles and clinical mastitis (Table 4.11).

### 4.3.2 Subclinical Mastitis

A total of 11,287 herd cultures were taken throughout the study on 3,286 different cows. The number of samples collected per cow varied from 0 to 7 with the average being 3.4 cultures. From the cultured milk samples, 4,363 showed no bacterial growth. An additional 5,354 cultures showed bacterial growth, but no growth of a known bacterial pathogen. There were 319 contaminated samples. The remaining 1,251 cultures showed growth of a bacterial pathogen. Of these cultures, 698 showed growth of a contagious pathogen, 553 of an environmental pathogen. The most commonly isolated bacteria were *Escherichia coli, Streptococcus dygalacia* and *Staphylococcus aureus* with 106, 344, 631 positive cultures, respectively.

For the analysis of association between the *BoLA-DRB3.2* genotypes and presence of a bacterial pathogen, the 1,251 cultures were treated as positive results. For the
analysis between BoLA genotype and presence of bacteria (not necessarily a bacterial pathogen), 6605 cultures were treated as positive (1251 positive cultures + 5354 cultures positive for bacterial growth) (Table 4.2).

For the presence of bacteria in herd cultures, the estimated heritability was 0, whereas for the presence of bacterial pathogens, the estimated heritability was 0.044.

Mixed model analyses were performed on both traits at $h^2=0$, 0.05 and 0.10 (Tables 4.4 and 4.5). The first trait, the number of cultures positive for the presence of bacteria, showed significant (p<0.05) effects for cultures, the covariate, as well as herd. Lactation was significant at the p<0.1 level. BoLA-DRB3.2 genotype was not a significant effect. The presence of bacterial pathogens as an indicator of subclinical mastitis demonstrated that cultures, herd and lactation were significant at the p<0.05 level. BoLA-DRB3.2 genotype was significant at the p<0.10 level.

DRB3.2*16 was contrasted against all others for both traits. The same contrasts were performed for DRB3.2*23. For the presence of bacteria, the contrasts showed no significant association between either allele DRB3.2*16 or DRB3.2*23 and the trait. For the presence of bacterial pathogen, there was no significant association between the trait and DRB3.2*16. DRB3.2*23, however, was significantly associated (p<0.10) with an increase in subclinical mastitis caused by bacterial pathogens (Table 4.11).

Mixed model analyses were also performed to investigate associations between BoLA genotype and the presence of each of the three most commonly isolated bacterial pathogens in the herd cultures (E. coli, S. aureus and Strep. spp.) at $h^2=0$, 0.05 and 0.10 (Tables 4.6, 4.7 and 4.8). For E. coli, only herd was significant (p<0.05). For S. aureus, the number of cultures, genotype, herd, and lactation were all significant effects (p<0.05).
This was also true for \textit{Strep.} spp. as the effects of number of cultures, genotype, herd and lactation were all significant to the p$\#0.05$ level.

Contrasts between alleles DRB3.2*16, *23, *16/23 and all others were performed for each of the three pathogens. DRB3.2*23 was shown to significantly increase the number of cultures positive for \textit{E. coli} (p$\#0.10$) and \textit{Strep.} spp. (p$\#0.05$). DRB3.2*16, however, was shown to decrease the number of cultures positive for \textit{Strep.} spp. (p$\#0.05$) (Table 4.11).

\textbf{4.3.3 Mastitis at Calving}

A total of 2143 samples were collected and cultured. Among these cultures, 1024 showed no bacterial growth. A further 761 showed no growth of a bacterial pathogen. For analysis, only the first calving of each cow was selected. A total of 1306 samples were used. From these, 633 showed no bacterial growth, and 458 showed no growth of a bacterial pathogen. Of the remaining 215 cultures, 65 contained contagious pathogens, while 150 contained environmental pathogens. The most commonly isolated bacteria were \textit{Pseudomonas} spp., \textit{Escherichia coli}, \textit{Staphylococcus aureus}, and \textit{Streptococcus dysgalactia} with 16, 41, 63 and 68 positive cultures, respectively.

For analysis of the association between BoLA genotype and the presence of bacteria at freshening, a total of 673 samples were treated as positive. For the association between \textit{BoLA-DRB3.2} genotype and the presence of bacterial pathogens at freshening, a total of 215 cultures were treated as positive (Table 4.2).

Heritabilities for both traits were estimated using VCE. In both cases, the estimated heritabilities were 0. Mixed analysis was performed on both traits at heritabilities of 0, 0.05 and 0.1 (Tables 4.9 and 4.10). For the analysis of the presence
of bacteria at freshening, only herd was significant at the p<0.05 level. Lactation number was significant at the p<0.1 level. BoLA-DRB3.2 genotype had no significant effect on this trait. For the mixed model analysis of the presence of bacterial pathogens at calving, herd and lactation were significant at the p<0.05 level. BoLA genotype had no effect.

Contrasts between allele DRB3.2*16 and all others showed no significant association between the allele and presence of bacteria at calving. Similarly, no significant association was seen between allele DRB3.2*23 and this trait. Similar results were observed for the contrasts between genotypes when considering bacterial pathogens: neither *16 nor *23 were significant (Table 4.11).

4.3.4 Somatic Cell Score and Production Traits

A general linear model analysis was performed on all selected traits. The results are presented in Table 4.9. Only EBV Milk was significant at the p<0.10 level. No other associations were detected between SCS or production traits and BoLA-DRB3.2 genotype.

Contrasts between allele DRB3.2*16 and all others showed only one significant association (Table 4.13). Allele DRB3.2*16 was significantly associated (p<0.10) with increased fat percent. No significant association was detected between allele DRB3.2*23 and any production trait.

4.4 Discussion and Conclusion

This study examines potential associations between two BoLA-DRB3 alleles commonly reported to have various influences on mastitis or SCS. Alleles DRB3.2*16 and *23 were examined for associations with clinical mastitis, subclinical mastitis,
mastitis at calving and overall SCS. Previous studies by Kelm et al. (1997), Starkenburg et al. (1997), Dietz et al. (1997), and Sharif et al. (1998) also looked at the effect of these alleles on disease traits, including mastitis. These studies, however, have produced conflicting results with respect to the nature of these associations. Kelm et al. (1997) and Dietz et al. (1997) found that DRB3.2*16 was associated with increased SCS, while Starkenburg et al. (1997) and Sharif et al. (1998) found that DRB3.2*16 was associated with a decrease in SCS in Holsteins. With respect to DRB3.2*23, Dietz et al. (1997) found that this allele was associated with an increased of acute SCS whereas Kelm et al. (1997) identified an association between the allele and decreased EBV for clinical mastitis and Sharif et al. (1998) found the association to be with increased susceptibility to clinical mastitis. Given that these two alleles have repeatedly been reported to be associated with various forms of clinical and subclinical mastitis, further investigation was necessary in order to reconcile the nature of these associations by studying a larger population with extensive health and production records. The present study failed to find any association between DRB3.2*16 and any measure of mastitis. The study did, however, support the findings of Sharif et al. (1998) that DRB3.2*23 is associated with increased susceptibility to mastitis, in particular subclinical mastitis caused by E. coli and Strep. spp. infection. The detection of clinical mastitis was dependent on the producers’ ability to identify cases, while the detection of subclinical mastitis was independent of the producers. For this reason, the results for subclinical mastitis may be more reliable than those for clinical mastitis.

A large number of milk samples were collected and cultured throughout the trial period, for the purpose of clinical case identification, as well as identification of
subclinical mastitis and mastitis at freshening. Noteworthy are the differences in the types of organisms cultured from each of the sample types (Table 4.2). Of the 475 positive samples from clinical mastitis episodes, 83.6% of the pathogens cultured were environmental pathogens, with only 16.4% of the pathogens contagious. This is consistent with the findings of Sargeant et al. (1998) who identified contagious pathogens in approximately 12% of milk samples collected from cows with clinical mastitis and environmental pathogens in approximately 88% of samples. Mastitis control programs have traditionally focused on the control of contagious pathogens transmitted during the milking process (NMC 1996). This emphasis has had a substantial impact on the relative frequencies of cases of mastitis caused by contagious and environmental pathogens (Smith & Hogan 1993; Fox & Gay 1993). The difference in frequencies seen in the Sentinel Herd may be even larger than is initially apparent. Clinical mastitis episodes caused by environmental pathogens such as coliforms, are often cleared very quickly, within 7 days of infection (Smith & Hogan 1993). While the cow may show signs of clinical mastitis, the organisms may have been cleared from the udder prior to the collection of the milk sample.

The culture results from clinical mastitis can be contrasted against those from the subclinical cultures. The routine herd cultures were more evenly divided between contagious and environmental pathogens. Of the positive cultures, 44.2% were environmental pathogens, 55.8% were contagious. It has been previously shown that contagious pathogens are often present in the udder at a sub-acute level for extended periods of time (Fox & Gay 1993). The results of this study agree with those findings. As expected, S. aureus was the predominant organism cultured from the herd samples. It
was present in 50.4% of all the positive cultures. Although mastitis control programs have been successful in decreasing the clinical mastitis caused by *S. aureus*, the need for further reduction, to prevent the development of subclinical mastitis, is evident.

Subclinical mastitis is perhaps as important as clinical mastitis because of the large production losses associated with the disease (Rajala-Schultz et al. 1999).

Results from the samples collected at calving were similar to those from the clinical cases. Contagious pathogens accounted for 30.2% of the positive cultures, while environmental pathogens accounted for 69.8%.

The $h^2$ estimated for all three mastitis traits (clinical mastitis, subclinical mastitis and SCS) are well within the published ranges. Estimates of clinical mastitis occurrence have ranged between 0 and 12%, with most estimates less than 10% (Emanuelson et al. 1988; Lund et al. 1994; Poso & Mantysaari 1996; Weller et al. 1992; Young et al. 1960).

The $h^2$ estimated in this study for the number of clinical mastitis cases was 9.0%. Few studies have attempted to estimate a $h^2$ for subclinical mastitis. Most estimates of this nature have been based on SCC, which has a range of $h^2$ between 5 and 25% (Rogers et al. 1991; Weller et al. 1992; Schutz et al. 1994; Reents et al. 1995, Poso & Mantysaari 1996). It is not surprising that the $h^2$ of 4.4% calculated in this study for the presence of a bacterial pathogen was somewhat lower than the published estimates based on SCS.

Few significant results were found in this study from the mixed model analysis. No associations of any type were found to exist between clinical mastitis and either allele $DRB3.2*16$ or $*23$. However, the associations between allele $DRB3.2*23$ and subclinical mastitis caused by pathogens in general ($p$<0.10), *E. coli* ($p$<0.10) and *Strep. spp.* ($p$<0.05) are consistent with the findings of Sharif et al. (1998) who found an association.
between allele *23 and increased susceptibility to clinical mastitis. No associations were seen between the incidence of mastitis at calving and either allele. For the SCS and production traits, only one association was found \((p\neq 0.10)\). Allele \(DRB3.2*16\) was found to be associated with an increased EBV for fat percentage. Sharif \textit{et al.} (1999) found an association between \(DRB3.2*8\) and increased fat yield. The study did not, however, find any associations between production traits and alleles \(DRB3.2*16\) and *23.

Several different factors may have contributed to the differences in findings between this, and previous studies. These factors may have included spurious associations, differences in allele frequencies, as well as different states of linkage disequilibrium between alleles within populations. These contradictory results point to the need for studies that examine the mechanisms that may influence MHC-disease interaction. The amino acid sequence within the antigen-binding groove may also play a role in the observed differences. For example, a study by Sharif \textit{et al.} (2000) investigated the association between amino acid motifs in the antigen binding groove and various alleles of the \textit{BoLA-DR} complex. The study found an association between a particular motif of pocket 4 and occurrence of clinical mastitis by \textit{Staphylococcus} spp. This motif, which contains a glutamic acid at position \(\exists 74\), is present in alleles \(DRB3.2*22, *23\) and *24. The same study showed tendencies for association between other amino acid substitutions within pocket 4 and mastitis. The presence of a charged residue at position \(\exists 13\) and the presence of arginine at \(\exists 71\) were also associated with \textit{S.} spp. infection. Each of these three motifs are found within pocket 4 of \(DRB3.2*23\), among others. Other investigators have also examined the amino acid sequence motifs at the antigen-binding groove for associations with disease traits (Kim \textit{et al.} 1985; Xu \textit{et al.} 1993).
Overall, the results from this study show no associations between $DRB3.2*16$ and any measure of mastitis. However, when the data from all previous studies are considered, it is likely that $BoLA-DRB3.2*23$, or alleles with similar binding pocket motifs, are contributing to mastits (clinical and/or subclinical) of Holsteins.