Chapter 2: Literature Review

2.1 Bovine Mastitis

2.1.1 Biology of the Disease

Mastitis is the most significant disease affecting dairy cattle (Shook and Schutz 1994). The economic, animal productivity, international trade and animal welfare issues associated with the disease make it of great importance to the agricultural industry (Owen et al. 2000). Mastitis is an inflammation of the mammary gland and primarily results from invasion of pathogenic organisms through the teat canal, resulting in loss of potential milk production in the affected quarter of the gland (Erskine 2001). This infection and inflammation results in both physical and chemical changes in milk. Bacteria and leukocytes in infected quarters release products, many of which are chemotactants for leukocytes. Neutrophils move rapidly from the blood into the secretions of infected quarters, causing an increase in the somatic cell count (SCC) of milk. Clinical cases of mastitis are characterized by the presence of one or more of the following symptoms: abnormal milk, udder swelling and systemic signs including an elevated temperature, lethargy and anorexia. Subclinical cases show no visible changes in the appearance of the milk or the udder, but milk production decreases, composition is altered and bacteria are present in the secretion (Erskine 2001). The greatest risk of acquiring mastitis occurs in the first 50 days of lactation (Sargeant et al. 1998) and in the early part of the dry period. The risk of clinical mastitis also increases with increasing parity (Sargeant et al. 1998).

Mastitis results once bacteria pass through the teat duct and multiply in milk-producing tissues causing an inflammatory response. Microorganisms may breach the
teat duct during the milking process, between milkings or during the dry period. Adherence of the bacteria to the tissues lining the interior of the mammary gland prevents them from being flushed-out during the milking process. The bacteria then produce virulence factors that cause swelling and the death of the milk producing cells. The toxins increase blood vessel permeability to blood plasma, promote the adherence of polymorphonuclear neutrophilic leukocytes (PMN) and attract PMN to the affected area. The PMN may engulf the bacteria or be killed by the invading organism. Both of these result in the production of additional substances that increase blood vessel permeability, increase the number of PMN and allow fluids and blood clotting factors to leak into the affected area. The influx of these substances constitutes the inflammatory response (NMC 1996). Once necrosis of the mammary stromal and parenchymal tissues has begun, there is a reduction in secretory function and potential milk production that may be irreversible.

The intramammary inflammatory response associated with mastitis not only results in a decrease in milk production, it also produces a decrease in quality of milk and manufactured products. It results in an increase of whey proteins, serum albumin, immunoglobulins, chloride, sodium, pH, free fatty acids and SCC in the milk. Mastitis also results in a reduction in synthesis of the main components of milk, such as lactose, fat, non-fat solids and casein (NMC 1996).

2.1.2 Infecting Organisms

Mastitis may be caused by both contagious and environmental pathogens. Contagious mastitis occurs with organisms that are spread from one infected quarter to another or between cows (often during the milking process). Environmental organisms,
however, are generally ubiquitous organisms in the housing environment (Erskine 2001). Infection may occur at any time, for example, milking, between milkings, during the dry period, or prior to the first calving. While proper hygiene can significantly reduce mastitis caused by contagious pathogens, clinical mastitis caused by environmental pathogens is of great importance for well-managed herds with low SCC (Smith & Hogan 1993). Environmental pathogens are a significant cause of IMI and clinical mastitis in heifers at calving. As management improves, the frequency of clinical cases due to contagious pathogens decreases and the relative frequency of clinical cases due to environmental pathogens increases (Smith & Hogan 1993; Fox & Gay 1993).

2.1.2.1 Contagious Mastitis

Contagious mastitis is defined as IMI transmitted directly from cow to cow (Erskine 2001). Incidence of contagious mastitis depends on the dose and type of microbes to which a cow is exposed as well as physical barriers and the innate and acquired defense mechanisms. Although many different types of bacteria may cause mastitis, those of greatest interest are the pathogens commonly found on dairy farms and those for which the prevalence of IMI is high. The main contagious organisms are *Streptococcus agalactiae*, *Staphylococcus aureus*, *Corynebacterium bovis* and *Mycoplasma* species (spp.). *S. aureus* is generally considered to be the most prevalent cause of IMI. It has been estimated that, depending on the breed and geographical location of the herd, between 7-40% of all cows are infected with *S. aureus* at any given time (Fox & Gay 1993).
2.1.2.2 Environmental Pathogens

The main environmental organisms are gram-negative bacteria, which include the coliforms and environmental streptococci. The gram-negative bacteria include *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Seratia*, *Pseudomonas* spp., *Proteus* and *Actinomyces pyogenes*. The environmental streptococci include *S. uberis*, *S. dysgaladiae*, and *S. equinus*. The majority of infections caused by coliform pathogens result in acute mastitis when compared to infections caused by contagious pathogens and environmental streptococci, but the infections are generally of shorter duration (less than seven days). The exception to this is *A. pyogenes*, which generates large and persistent production losses (Smith & Hogan 1993). The environmental streptococcus infections generally last less than 30 days (Smith & Hogan 1993). *E. coli* and *Klebsiella* are the most commonly isolated environmental bacteria. Approximately 80% of gram negative IMI result in clinical mastitis, whereas 50% of environmental streptococci infections result in clinical mastitis (Smith & Hogan 1993).

2.1.3 Economics of the Disease

Mastitis is the most costly disease of dairy cows. The costs associated with the disease include discarded milk, early culling, drug costs, veterinary costs, increased labour and most importantly, decreased quantity and quality of milk and manufactured products (DeGraves & Fetrow 1993). There are many different ways of estimating milk production losses from mastitis. Calculations can be made based on milk SCC, between-herd yield comparisons, between-cow yield comparisons, within-udder yield comparisons, within-cow yield comparisons and comparisons between identical twins.
All of these techniques have a degree of inherent bias that, in general, under-estimates the actual milk production loss that has occurred (DeGraves & Fetrow, 1993). A comparison of the yield of mastitic cows with that of healthy cows may not be the most appropriate approach to the problem of estimating the effect of mastitis on milk yield because cows that are higher producing are more likely to develop IMI (Rajala-Schultz et al. 1999).

Decreased milk production accounts for 75% of the production losses due to subclinical mastitis (Colleau & Le Bihan-Duval 1995). Subclinical mastitis may be economically more important than actual clinical mastitis due to the widespread losses associated with subclinical infection. Total milk loss in quarters affected with subclinical mastitis is approximately 10-26% (DeGraves & Fetrow 1993). Clinical mastitis results in the greatest losses when the case occurs early in lactation (DeGraves & Fetrow 1993). Cows in later lactations show a greater decrease following clinical mastitis than first lactation cows (DeGraves & Fetrow 1993). A quarter that experiences clinical mastitis may never completely recover, creating a carryover loss, although the carryover effect of mastitis and high SCC from one lactation to the next has been found to be statistically significant but small (Fetrow et al. 1991). The type of organism causing mastitis seems to be a minor factor influencing total production loss (Fetrow et al. 1991).

Economic losses have been calculated to be approximately $200 per cow per year or $2 billion annually (U.S.) (Nash et al. 2000; DeGraves & Fetrow 1993; NMC 1996). Approximately 10% of total value of milk sales is lost each year as a result of decreased milk production, increased milk replacement cost, discarded milk, drug costs, veterinary fees and labour costs (DeGraves & Fetrow 1993; NMC 1996). The cost of clinical
Mastitis has also been estimated to be $107 US per clinical episode with over 70% of the cost associated with decreased milk production and milk withheld from the market, over 20% with drugs, veterinary costs and replacement costs, and the remainder with labour (Smith & Hogan 1993; DeGraves & Fetrow 1993; NMC 1996).

**2.1.4 Treatment and Control**

The current methods of prevention and control of mastitis include proper milking hygiene, reduced exposure to environmental pathogens, dry cow antibiotic therapy, culling and therapy of clinical mastitis cases. Mastitis reduction can be achieved by decreasing teat-end exposure to pathogens or by increasing the resistance of cows to IMI (Smith & Hogan 1993). The exposure can be reduced by improving the dry cow and close-up heifer housing, calving area, lactating cow housing, bedding materials, ventilation, milking parlor and milking time hygiene.

Studies have indicated that resistance may be improved through diet, genetics, and to a lesser extent, vaccination. Through diet, improved resistance may be achieved by increasing vitamin E, selenium, vitamin A and β-carotene (Smith & Hogan 1993; NMC 1996). Vaccination as a whole, has contributed little to mastitis resistance or control (Smith & Hogan 1993). Even the new *E. coli* J5 vaccines are of limited efficacy (NMC 1996). They have only been shown to reduce the incidence and severity of clinical coliform mastitis during early lactation. They do not, however, reduce the percentage of mammary glands with infection and have no effect on contagious pathogens or environmental streptococci. The use of a *S. aureus* bacterin has been successful in reducing *S. aureus* infections in some, but not all herds (Fox & Gay 1993).
Currently, genetic strategies to improve resistance to mastitis include selection for lower somatic cell counts (NMC 1996) and enhanced immune responses (Wagter et al. 2000).

### 2.1.5 Somatic Cell Count

SCC refers to the PMN and other cells attracted to the mammary gland in large numbers during an infection. These cells include macrophages, leukocytes and neutrophils. Leukocytes are produced in larger numbers by the immune system in response to an infection and serve to eliminate the infecting organism. Their presence in the udder causes them to be secreted into the milk, altering the composition of the milk. Decreased SCC has been shown to improve dairy product quality, shelf-life and cheese yield (NMC 1996). The presence of somatic cells in large numbers in the milk is also an indication of clinical or subclinical infection.

SCC has become an important tool in selection. It has been included in the breeding goal and genetic evaluations are routinely published in several countries (Schutz 1994; Reents et al. 1995; Mrode and Swanson 1996; Boichard and Rupp 1997). Currently, it is used as an indicator of the presence of mastitis in individual cows as well as the entire herd. SCCs have been used as the selection criteria for a variety of reasons. Firstly, they are routinely recorded in most milk recording systems, and as such, are available on a large scale at a low cost. Secondly, SCC has a higher heritability (0.05-0.25) than clinical mastitis (<0.10), allowing genetic progress to be more easily made (Young et al. 1960; Emanuelson et al. 1988; Rogers et al. 1991; Weller et al. 1992; Lund et al. 1994; Schutz et al. 1994; Reents et al. 1995; Poso & Mantysaari 1996). Finally, the genetic correlation between SCC and clinical mastitis has been shown to be between 0.6
and 0.8 (Shook and Schutz 1994). This relatively high genetic correlation indicates that progress can be made in the reduction of mastitis by selecting for decreased SCC.

Concern has been raised by some scientists, however, that continually decreasing SCC by selection, could impair the capacity for leukocyte recruitment and the ability to respond to IMI (Schukken et al. 1994). As leukocytes in the udder are present to resolve the IMI, a very low SCC may predispose cows to a risk of clinical mastitis (Schukken et al. 1994; Suriyasathaporn et al. 2000). Cows with very low SCC ($\leq 150,000$ cells/ml) may be more susceptible to clinical mastitis, whereas quarters with moderate to high initial SCC (400,000 to 600,000 cells/ml) have a lower risk of being infected following experimental challenge with mastitis pathogens (Kehrli & Shuster 1994).

In contrast, Rupp and Boichard (2000) found no increase in susceptibility to clinical mastitis for cows with very low SCC. In fact, cows with the lowest SCC at the initial test in the first lactation had the lowest probability of being infected by clinical mastitis later in first lactation or at the beginning of second lactation. The discrepancies between these findings suggest that an alternative method of selection for increased resistance to IMI would be desirable. Selection based on quantitative trait loci (QTLs) associated with the disease of interest may be an alternative.
2.2 Breeding for Disease Resistance

Traditional breeding goals for dairy cattle have focused mainly on increasing productivity. The intense selection for traits such as milk yield, may have led to an increase of disease in the population (Lucey et al. 1986; van Dorp et al. 1999; Fleischer et al. 2001). As a result, researchers have begun to examine the benefits of including disease resistance, or related traits, in the selection index. There are many reasons to include disease resistance in a breeding program. These include production losses, unfavourable genetic correlation between productivity and disease, increased resistance to anti-microbial drugs, animal welfare issues and positive epidemiological feedback due to decreased disease transmission when the proportion of resistant animals increases in the population (Detilleux 2001). Production losses include both the direct costs such as decreased or wasted output, the indirect costs such as the prevention and treatment of the disease, as well as other costs including trade losses when diseases such as Foot and Mouth Disease (FMD) are present.

Another approach that is being examined is selection for improved general immune response. Animals that are greater immune responders may be better prepared to resist or tolerate many diseases as opposed to an animal selected for resistance to a specific disease. Wagter et al. (2000) developed a method to classify Holsteins based on phenotypic variation of serum antibody response to various test antigens. While the results do not demonstrate a conclusive relationship between antibody responsiveness and decreased risk of peripartum mastitis, they do indicate that higher levels of antibody may be beneficial in some herds.
Increased antibiotic resistance is a growing concern among members of the agricultural community as well as the public. Resistance to traditional antimicrobial agents has increased over the last few decades due, in a large part, to the problems of both over-use and under-use (Detillieux 2001). Over-use refers to the increased use of antibiotics to treat infection as well as the use of antibiotics as growth promoters. As a result, normal flora and pathogenic microorganisms are routinely exposed to the antibiotics creating selective advantage to resistant strains. Under-use refers to improper use of antibiotics. Inadequate antibiotic concentration or use for an insufficient period of time leads to the elimination of only the sensitive organisms and the proliferation of the more resilient.

Another possible benefit of breeding disease resistant animals is the creation of herd immunity. If most of the animals in a population are resistant to infection, particularly one caused by a contagious pathogen, the chances of two susceptible individuals coming in contact with one another greatly decreases, thus preventing the spread of the disease (MacKenzie & Bishop 2001).

Mastitis is an excellent example of a disease for which selective breeding could improve resistance. Mastitis has an elevated incidence of approximately 20-40% per cow-year; the costs, both direct and indirect, associated with mastitis are high; and there is a relatively strong and unfavourable genetic correlation between milk production and mastitis (Heringstad et al. 2000). Strandberg and Shook (1989) demonstrated that breeding for increased production under the traditional progeny testing programme, without selection for resistance to mastitis, results in a genetic increase of 0.02 cases of mastitis per cow per year (assuming a genetic correlation between mastitis and milk yield.
Breeding for mastitis resistance would also improve the total economic merit, reduce the need for antibiotics and vaccination as well as address ethics and animal welfare issues.

There are several ways by which disease resistance in a population may be improved through selective breeding. Selection can be based on either phenotype (such as occurrence of disease) or genotype (marker or QTL associated with the disease) and may be either direct or indirect.

### 2.2.1 Phenotypic Selection

To date, most genetic progress for quantitative traits in livestock has been made by selection on phenotype or on estimated breeding values (EBV) derived from phenotype, without any knowledge of the number or nature of genes that affect the trait.

#### 2.2.1.1 Direct Phenotypic Selection

The calculation of heritabilities ($h^2$) and EBVs for economic traits, including disease resistance enables parents to be selected for the next generation of breeding. This procedure is difficult, however, with respect to disease resistance, as the heritabilities of disease traits tend to be very low (≤10%) (Shook 1989; Uribe 1995). There are many examples of direct phenotypic selection. These include trypanosomiasis in cattle (Naessens et al. 2002) and the direct selection of dairy bulls for mastitis resistance of their daughters (Groen et al. 1997). Trypanosomiasis is an interesting example as the current selection strategies are based solely on selecting animals that are tryptotolerant and consequently, not truly resistant to the disease. Researchers are currently working towards genetic strategies for evaluating resistance to this disease.
2.2.1.2 Indirect Phenotypic Selection

Indirect selection refers to selection applied to a trait that is correlated to the trait of interest. Genetic change following indirect selection depends on the intensity of selection, the $h^2$ of both the marker and desired trait and the correlation between the two traits. Marker traits are most useful if the marker trait can be measured in both sexes, has a higher $h^2$ than the disease trait, indicates subclinical variations in the disease, is measurable early in life and at a low cost (Falconer & Mackay 1996).

One of the best examples of indirect phenotypic selection in dairy cattle is the use of SCC to select against occurrence of clinical mastitis. A genetic correlation between SCC and incidence of clinical mastitis has been demonstrated (Shook 1989; Shook & Schutz 1994).

2.2 Genotypic Selection

Quantitative genetic approaches have been very successful and substantial rates of genetic progress have been, and continue to be, achieved for production traits. These methods, however, ignore the additional genetic progress that can be made based on information about specific genes involved in the trait of interest. Molecular genetics is providing a great deal of information about genes at the DNA level. Genotypic selection may allow this information to be used to enhance genetic progress of quantitative traits.

2.2.1 Direct Genotypic Selection

Direct genotypic selection is based on the genotype of an animal for a locus or genetic system (e.g. the Major Histocompatibility Complex (MHC)) affecting the trait of interest. This technique is most useful when the disease is controlled by one or a few
genes, as is the case for porcine stress syndrome (Rempel et al. 1993). This type of selection is often used for simply inherited effects or defects such as mulefoot (Leipold et al. 1998), bovine leukocyte adhesion deficiency (Shuster et al. 1992) and deficiency of uridine monophosphate synthase (Poli et al. 1996; Schwenger et al. 1993).

2.2.2.2 Indirect Genotypic Selection

Similar to indirect phenotypic selection, indirect genotypic selection makes use of a trait that is correlated to the trait of interest. Indirect genotypic selection, however, assumes that the genotype of the locus affecting the indicator trait also affects the trait of interest. This method of selection is useful if the marker trait is of higher $h^2$ than the trait of interest and there is a high genetic correlation between the indicator trait and the trait of interest. Selection based on quantitative trait loci (QTL) associated with the trait of interest is an example of indirect genotypic selection.

QTLs may be found using genome-wide scans using anonymous molecular markers such as amplified fragment length polymorphism (AFLP). QTL are often discovered, however, based on known physiological and/or biochemical information about a particular gene or gene region. If a particular region is known to influence character expression, it may be tested directly, at the population-level, for associations between the trait and specific alleles at loci in the region. Genome-wide scans have revealed several putative QTL for SCC and mastitis on chromosomes 3, 4, 6, 14, 23 and 27 in cattle (Heyen et al. 1999; Klungland et al. 2001). No effects reaching genome wide or suggestive significance thresholds were found for SCC, but the putative QTLs on chromosome 23 were nearest to the significance thresholds. The MHC is located on
chromosome 23. For this reason and the fact that the MHC is known to play a critical role in immune response, the MHC is a promising candidate gene for many disease traits.
2.3 Major Histocompatibility Complex (MHC)

2.3.1 History of the MHC

The MHC is a tightly linked cluster of genes in which the majority of products are associated with host defense and intercellular communication. The genome of all mammalian, avian, amphibian and fish species studied to date, contains MHC genes (Klein 1986). The discovery of the MHC resulted from research of R.A. Gorer and G.D. Snell in the 1930s. Using inbred strains of mice to identify blood-group antigens, Gorer identified four groups of genes that encoded blood-cell antigens (designated I-IV) (Gorer 1937). The MHC was further characterized by Snell in transplantation studies in congenic mouse lines. Snell revealed that the group II antigens were involved in the rejection of transplanted tumours and other tissues. These genes were designated as the “histocompatibility-2” (H-2) genes, in reference to Gorer’s group II blood-group antigens (Snell 1948). Following the discovery in mice, the MHC was also identified in chickens (Schierman & Nordskog 1961), humans (1963, see Klein 1986), swine (Vaiman et al. 1970; Viza et al. 1970) and cattle (Amorena & Stone 1978; Spooner et al. 1978).

2.3.2 Genetics of the MHC

The MHC is a relatively large cluster of genes, spanning nearly 4 Mb of the human genome, approximately 2.5Mb of the cattle genome, and 2 Mb of the mouse and porcine genome (Lewin 1996; Rothschild et al. 2000). It is located on chromosome 6 in humans, chromosome 17 in mice, chromosome 7 in swine and chromosome 23 in cattle. The human MHC is commonly referred to as the human leukocyte antigen (HLA) complex, the mouse MHC as the H-2 complex, the swine MHC as the swine leukocyte
antigen (SLA) complex and the cattle MHC as the bovine leukocyte antigen (BoLA) complex. This nomenclature is somewhat misleading, however, as not all of the genes of the MHC actually encode leukocyte antigens. The human and mouse MHC have been estimated to contain more than 200 genes, pseudogenes and gene fragments and this is likely similar for most mammals (The MHC sequencing consortium 1999).

The genes of the MHC are organized into three distinct classes in mammals (class I, class II and class III). Avian species also contain a fourth class of genes, although its function is not yet completely clear (Rothschild et al. 2000). Each of these classes is divided into regions and subregions, which contain a number of genes and/or pseudogenes. The classical class I (class Ia) genes often consists of the A, B and C genes. Several nonclassical class I (class Ib) genes have also been identified (E, F, G, H, J, and MIC), but these are generally less polymorphic and expressed at lower levels than the class Ia MHC genes. Class II MHC genes can also be designated as classical and nonclassical. The classical class II (class IIa) genes generally include the DR, DP and DQ genes. The nonclassical (class IIb) genes primarily encode the DM, DN and DO molecules. Other genes of interest located within the class II region are the low molecular weight polypeptide proteasome subunits (LMP2 and LMP7), transport associated proteins (TAP1 and TAP2) genes. The class III region contains a heterogeneous collection of more than 30 genes including several complement components (C2, C4A, C4B, Bf), β-21 hydroxylase, tumor necrosis factor (TNF-α and TNF-β) and heat shock protein genes (HSP70) (Milner & Campbell 2001).
2.3.3 Function of the MHC

The MHC plays a central role in the development of both antibody and cell-mediated immune responses. The MHC class I and II genes code primarily for cell surface glycoproteins that bind and present peptides to T-lymphocytes (T-cells) of the immune system, which results in T-cell stimulation and activation. T-cells generally recognize foreign antigens in association with self MHC molecules (Zinkernagel & Doherty 1979). Because MHC molecules function as antigen presenting structures, the particular set of MHC molecules expressed by an individual influences the repertoire of antigens to which individual T-helper (Th) cell and T-cytotoxic (Tc) cells can respond. For this reason, the MHC partly determines the response of an individual to antigens of infectious organisms and the MHC has, therefore, been implicated in susceptibility and resistance to infectious disease and in the development of autoimmunity (Lewin 1996).

MHC class I glycoproteins are present on most nucleated cell types and are involved in Tc cell antigen recognition and activation (Zinkernagel & Doherty 1979). MHC class II glycoproteins are found primarily on antigen presenting cells (APC) such as B-cells, macrophages, thymic epithelial cells, human activated T-cells and dendritic cells, for example, Langerhans cells of the skin (Kappes & Strominger 1998). Class II glycoproteins are also involved in the presentation of antigenic peptide fragments to T-cells, resulting in Th cell activation. Differences between the MHC class I and class II molecules can also been seen in the peptide-binding domain. While the peptide-binding cleft in class I molecules is closed at both ends, the class II molecules have an open binding cleft. As a result, class I molecules are only able to accommodate short peptides containing an average of nine amino acid residues. On the other hand, class II molecules
are able to accommodate much larger peptides of 13-18 amino acids. The peptides, in both class I and class II molecules, contain specific amino acid residues (anchor residues) that help to anchor peptide into the groove of the MHC molecule. The third class of genes (class III), generally encode secreted proteins associated with host defense, including soluble serum proteins, components of the complement system and tumour necrosis factors.
2.4 Bovine Leukocyte Antigen (BoLA)

The MHC of cattle was first discovered by Amorena and Stone (1978) and Spooner et al. (1978). The term BoLA was defined at the First International Bovine Leukocyte Antigen workshop (Spooner et al. 1978) and refers to both the *Bos taurus* and *Bos indicus* species.

2.4.1 Genetics of BoLA

The BoLA complex is located on chromosome 23 at band 22. It is organized into three classes (I, II and III), each with genes encoding proteins with distinct tissue distribution and function. The general organizational features of the BoLA complex have been determined by analysis of somatic cell hybrids, radiation hybrids, linkage, and fluorescent in situ hybridization (FISH) (Andersson et al. 1988; Skow et al. 1988; Band et al. 1998, 2000). In contrast to the single cluster of genes that comprise the typical mammalian MHC, BoLA genes are found in two separate regions of chromosome 23 (Andersson et al. 1988; van Eijk et al. 1993). The larger gene cluster (~2.5Mb) located at BTA23 band 22 contains all of bovine class I (*BoLA-A*, *BoLA-B*, *MOG*) and class III (*TNFα*, *TNFβ*, *BF*, *C4*, *CYP21*, *HSP70-1*, *HSP70-2*, *EAM*) genes, as well as genes encoding both subunits of the classical class IIa proteins DQ and DR (*DQA1*, *DQA2*, *DQB1*, *DQB2*, *DRA*, *DRB1-3*). The remaining class IIb loci (*DIB*, *DNA*, *DOB*, *DYA*, *DYB*, *TCP1*, *LMP2*, *LMP7*, *TAP2*) are located in a smaller cluster near the centromere at BTA bands 12-13 (Lewin 1996). This arrangement is also seen in sheep, goats and deer and likely represents an ancient evolutionary event. Comparative analysis of the gene order in BoLA with the human MHC, which is found as a tightly linked cluster, revealed
that the disruption of BoLA likely was caused by a single large chromosomal inversion (Band et al. 1998).

### 2.4.1.1 BoLA Class I Genes

The BoLA complex contains at least 28 class Ia sequences (Lindberg & Andersson 1988; Rothschild et al. 2000). Serologically, three of these genes have been shown to be expressed transcriptionally in bovine lymphocytes (Joosten et al. 1988; Ellis et al. 1992; Garber et al. 1994). The genes act as restriction elements for CD8+ cytotoxic T-cells. In total, more than 50 BoLA antigens have been recognized, nearly all of which behave as alleles of a single locus. At the 5th BoLA workshop held in 1992, 27 of these antigens were designated as full-status BoLA specificities, 25 as provisional workshop (w) specificities (Davies et al. 1992).

### 2.4.1.2 BoLA Class II Genes

The genes of the BoLA class II region have been divided into two subregions designated class IIa and IIb. The first subregion (IIa), located at BTA23q22, contains the DR and DQ genes, many of which have been shown to be expressed on the cell surface (DQA, DQB1, DQB2, DRA, DRB3) (Lewin 1996). Cattle express one DR pair (DRA & DRB3) and one or two DQ gene pairs per haplotype. The second subregion (IIb), located at BTA23q12-13, contains the DIB, DOB, DNA, DYA, DYB, DMA and DMB, LMP2, LMP7, and TAP1 genes (Andersson et al. 1988; Andersson & Rask 1988). Thus far however, evidence for expression of these genes can only be found for LMP2 (Lewin 1996).
2.4.1.3 BoLA Class III Genes

The genes of the BoLA class III include components of the complement system (Bf, C2, C4A, C4B), heat shock proteins (HSP70), steroid hydroxylases (CYP21, β-21 hydroxylase), and tumour necrosis factors (TNFα and TNFβ).

2.4.2 BoLA Typing

2.4.2.1 Class I Typing

Class I BoLA genes and products have been studied using a variety of methods that include serology and electrophoretic methods (Caldwell et al. 1977; Amorena & Stone 1978; Spooner et al. 1978; Joosten et al. 1988). Serology was the most successful of these methods, and led to the initial characterization of the BoLA class I system. Both of these methods, however, are expensive, labour intensive and require the use of viable cells. BoLA-A and BoLA-B typing also require a large range of specific antisera, generally derived by skin grafting between animals or immunization with allogenic lymphocytes. The sera commonly employed for BoLA-A typing may not be specific for allele products of a single locus, and may contain antibodies to the products of other class I loci. Thus, BoLA-A typing may define a class I haplotype rather than an allele (Lewin 1996; Ellis et al. 1996, 1999).

More recently, Ellis et al. (1998) described the DNA-based typing of BoLA class I genes using sequence-specific amplification of expressed class I alleles from cDNA. This method is still limited, however, by the lack of information on class I allele sequence variation between breeds, and by breed-specific differences in the class I types represented (Bull et al. 1989).
The development of an improved DNA-based typing method for BoLA class I genes would permit further study of the relationship between MHC and disease, at the population level. The development of such a technique is complicated, however, by the expression of multiple class I genes in most haplotypes and the lack of obvious sequence differences between these genes (Ellis et al. 1999). The design of primer sequences, then, is not locus-specific, but limited to the available DNA sequences.

Sawhney et al. (2001) developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for BoLA class I typing. The polymorphic region containing exon 2 and 3 is amplified from genomic DNA using primers design from published BoLA class I sequences. The 700 bp fragment generated by the PCR incorporates all of the polymorphisms observed in the published sequences. The amplified product is then digested with two different restriction enzymes (TaqI and Ddel). It was found, however, that additional restriction enzymes may be required to reveal further class I polymorphisms. Suggested enzymes include RsaI, HinfI and Hhal. This method could be used, in relation to disease susceptibility and resistance, to determine linkage between phenotype and RFLP.

2.4.2.2 Class II typing

Historically, several different methods have been applied for the typing of class II BoLA genes. These methods have included T-cell typing with T-cell lines and clones (Teale & Kemp 1987; Rothel et al. 1990); RFLP typing (Sigurdardottir et al. 1988); typing by immunoprecipitation and isoelectric focusing (IEF) (Joosen et al. 1989); serology (Mackie & Stear 1990; Arriens et al. 1991; Davies & Antczak 1991b; Williams et al. 1991); mixed lymphocyte culture (MLC) typing (Davies & Antczak 1991a);
heteroduplex analysis (Sitte et al. 1995) and sequence-specific oligonucleotides (Sitte et al. 1996). These techniques tend to be expensive and labour intensive. They are also limited in their ability to characterize different BoLA haplotypes. As a result, several different techniques for characterizing the BoLA class II genes have been developed. While DNA sequencing remains the “gold standard” for characterizing polymorphisms within the BoLA class II genes, this laborious procedure is of limited usefulness in defining large populations. As BoLA-DRB3 exon 2 is the most variable region of the class II alleles, most of the typing methods available focus on the ability to detect polymorphisms within this peptide-binding region.

2.4.2.2.1 BoLA-DRB3 Typing Methods

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

The availability of sequence data for HLA genes has been instrumental in the development of new sequence-based HLA typing techniques. As sequence data for the BoLA genes has become more readily available, HLA techniques have been modified for use in BoLA typing. One such technique, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), has been used to study polymorphism within the BoLA-DRB3 gene (van Eijk et al. 1992). This PCR-RFLP consists of two stages, a hemi-nested PCR amplification and a restriction enzyme digestion of the amplified fragment. The PCR amplification produces a 284 bp fragment of exon 2 of the DRB3 gene. The fragment is then digested separately with three different restriction enzymes (Rsal, HaeIII and BstYI). These fragments are separated on a polyacrylamide gel. The combination of the banding patterns, from digestion with each of the enzymes, determines the genotype of the individual. Initially, this method was able to detect 30 different BoLA-DRB3
alleles. To date, 50 different types have been identified in exon 2 of DRB3, but not all the types have a corresponding sequenced allele (Lewin et al. 1999). While a powerful technique, certain drawbacks are associated with this method. Firstly, PCR-RFLP is only able to detect a fraction of the known polymorphism as it can only identify polymorphisms that create or destroy a restriction enzyme site. Secondly, it requires several laborious and time-consuming steps.

**Denaturing Gradient Gel Electrophoresis**

Denaturing gradient gel electrophoresis (DGGE) is a method that attempts to reduce the time and labour involved in the direct sequencing of BoLA-DRB3 alleles by removing the need for cloning (Aldridge et al. 1998). Locus-specific primers are used to amplify a 240 bp fragment of exon 2 of BoLA-DRB3. One of the primers also contains a G-C rich clamp that improves the physical separation of the alleles by DGGE. The denaturing gradient gel can separate DNA molecules with minor differences in their nucleotide sequence by exploiting nucleotide sequence-dependent differences in DNA melting temperatures. The apparent optimum gradients are 10-15% acrylamide in parallel with a 15-60% denaturing gradient. Following separation, the gel is stained and the bands excised from the gel. PCR can then be performed on the eluted DNA to remove the G-C clamp, and to add an extension for fluorescent-based sequencing. The product can then be sequenced without the need for cloning. One definitive drawback to this method is that certain allele combinations may be difficult to separate. An individual containing different alleles of a similar melting temperature could appear to be homozygous at the BoLA-DRB3 level.
Sequence-based Typing

Based on the sequence-based typing (SBT) method developed for the HLA, PCR-SBT was designed to improve the resolution and accuracy of BoLA-DRB3 typing (Takeshima et al. 2001). Using the 88 known BoLA-DRB3 alleles, the predicted amino acid sequences of the $\beta_1$ domains encoded by these alleles were aligned. The $\alpha_1$ and $\beta_1$ domains are the portions of the polypeptide chains of class II MHC molecules that make up the antigen-binding groove. The variability seen in the $\beta_1$ domain was mainly concentrated in four hypervariable region pockets that are involved in the binding of antigens. The sequences could then be divided up into eight groups of alleles according to the amino acid sequences in the first hypervariable region. Following an initial PCR reaction to amplify exon 2 of the BoLA-DRB3 gene, PCR using the group-specific primers separated the alleles into their groups. The PCR product was then separated by electrophoresis on a 2% agarose gel and sequenced. One potential drawback of this method is that it does not provide the entire sequence of exon 2. It relies on PCR-based generation of each template using amplification primers located at the 5’ end of exon 2 encoding the first hypervariable region of BoLA-DRB. As a result, potential variations in sequence in these regions might remain unexamined and new alleles might be overlooked. Since the development of this technique, a further 15 alleles have been identified by PCR-SBT to bring the total of known BoLA-DRB3 alleles to 103 (Takeshima et al. 2002). Like DGGE, PCR-SBT is rapid and facilitates the sequencing of BoLA-DRB3 alleles without cloning.
2.5 Specific Allele Detection Techniques

PCR is a method that typically uses two oligonucleotide primers to amplify a DNA segment. Simple modifications of this method can be used to detect single base pair changes in genomic DNA. The introduction of PCR as a technique to identify specific alleles dramatically decreased the time and labour required to genotype a large population for those alleles. Despite this revolution, however, many of the techniques for the detection of specific alleles still required several complicated steps, including digestion with restriction endonucleases. One of the fundamental problems with digestion with restriction endonucleases is that detection is limited to polymorphisms that either create or destroy a restriction site. In order to rapidly screen a population for the presence of an allele, the development of a new method was necessary.

2.5.1 PCR Amplification of Specific Alleles

PCR amplification of specific alleles (PASA) was developed to facilitate the rapid detection of single base pair changes in genomic DNA by using specifically designed oligonucleotides (Sommer et al. 1989; Sarkar et al. 1990). This method is also known as allele-specific amplification (ASA), allele-specific PCR (ASP), and amplification refractory mutation system (ARMS) (Newton et al. 1989; Nichols et al. 1989; Wu et al. 1989). The principle is to design oligonucleotide primers that will preferentially amplify one allele over another. It is based on the fact that oligonucleotides with 3’ mismatch residues will not function as primers in PCR under appropriate conditions. PASA involves the amplification with two oligonucleotide primers such that one is allele-specific. The other primer contains a mismatch at, or near the 3’end. As a result, the
desired allele is efficiently amplified whereas the other is poorly amplified. Using this technique, a sequence of DNA containing the polymorphism of interest is amplified using two outer primers. This amplified product is then diluted a million fold. The alleles of interest are then detected using a nested PCR with one of the outer primers and two allele specific primers. The sizes of the fragments produced, when separated by agarose gel electrophoresis, determine the genotype of the individual. There are however, several shortcomings with this technique: 1) the specificity of PASA can be overwhelmed by high concentrations of template DNA; 2) two PCRs are required to determine whether an individual is homozygous or heterozygous for the allele of interest; 3) if the specific allele is absent and spurious bands of other sizes are not produced, another set of compatible primers must be added to generate a constant band that serves as an internal control for PCR; and 4) genes with more than two alleles per locus can not be efficiently detected with this method.

2.5.2 PCR Amplification of Multiple Specific Alleles

PCR amplification of multiple specific alleles (PAMSA) is based on modifications to the PASA technique that enable zygoty to be determined in a single reaction (Dutton & Sommer 1991). This is useful in situations in which two alleles are commonly observed. Three primers are combined in one reaction, instead of four primers in two separate reactions, as is the case with PASA. One allele-specific primer is synthesized to be longer than the other allele-specific primer by the addition of at least 30 non-complementary bases. The difference in size of the resulting amplification products can be detected by agarose gel electrophoresis. This allows both alleles to be detected in one reaction. Also in contrast to PASA, amplification products are always produced,
eliminating the need for an additional set of internal control primers. The competition of
the two allele-specific oligonucleotides can also prevent the high concentration of
template DNA from overwhelming specificity.

2.5.3 Tetra-Primer PCR

Tetra-primer PCR is designed to accomplish ASA in a single PCR, using two
different annealing temperatures and four different primers (Ye et al. 1992). Two
flanking primers amplify a specific gene or region. Two internal, allele-specific primers
amplify the specific alleles. The mismatch in the internal primers is located in the middle
of the oligonucleotide, as opposed to the 3’ end. The melting temperatures (Tm) of the
flanking primers are designed to be at least 10°C higher than those of the internal primers.
This is usually achieved by designing longer flanking primers. The first set of
amplification cycles at the higher temperature creates a small DNA fragment from the
flanking primers. The second set of cycles at the lower temperature makes use of this
fragment as a template for the inner primers. The resulting fragments produced depend
on the genotype of the individual. Two DNA fragments of different sizes are generated
in heterozygotes, but only one in the case of homozygotes. The internal fragments are
not centred in the fragment generated by the flanking primers. The two DNA fragments
created in the second program are of unequal length.

2.5.4 Bidirectional PCR Amplification of Specific Alleles

Bidirectional PCR amplification of specific alleles (Bi-PASA), developed by Liu
et al. (1997), is very similar in theory to the tetra-primer PCR method with a few
modifications: 1) In tetra-primer PCR, the mismatch is located in the middle of the inner
primers. In Bi-PASA, the mismatch is at (or near) the 3’ end. 2) The Bi-PASA inner
primers have short complementary segments and G+C rich tails. The tail serves to
efficiently switch from template-based amplification to self-amplification and to prevent
mega-priming. Mega-priming occurs when a DNA fragment generated by PCR acts as a
primer for a larger template in subsequent reactions. 3) While tetra-primer PCR uses two
annealing conditions, Bi-PASA uses a constant annealing temperature. 4) The inner
primers of tetra-primer PCR are 35 times more concentrated than outer primers. The
primers in Bi-PASA are of similar concentration. The primers in Bi-PASA consist of
two outer primers (P&Q) and two inner primers (A&B). Primers P and Q anneal at
different distances from the polymorphism to differentiate downstream and upstream
PASA assays on an agarose gel. Up to three different segments are produced during the
PCR reaction. PQ is always produced and serves as an internal control for the reaction.
PB and AQ fragments are also produced by the heterozygote. One of PB and AQ are
present in the homozygote. The Bi-PASA reaction consists of two distinct parts, the
template transfer amplification and self-amplification. The amplification conditions are
designed to favour self-amplification. The main drawback to Bi-PASA is that
interactions among the primers can influence yield and specificity.
2.6 Summary

The enormous economic losses associated with mastitis present a substantial problem for many producers. Although current control programs have made some progress toward reducing the frequency of IMI caused by contagious pathogens, many of the most acute cases of clinical mastitis are caused by environmental pathogens.

Selection for decreased occurrence of mastitis has been challenging. As with most disease traits, mastitis has a low heritability making genetic progress difficult (Shook et al. 1993). Selection based on an indicator trait such as SCC with a higher heritability has been more successful. There is debate, however, as to whether SCC is an appropriate indicator of mastitis as animals with chronically low SCC may be more susceptible to disease in general. Genetic methods of reducing occurrence of mastitis, through detection of QTLs associated with the disease or by selection for improved immune responses, may prove more useful.

The MHC region provides a relevant place to search for QTLs associated with disease, as the genes in this region are known to play an important role in regulation of immune responses. The BoLA-DRB3 gene region, in particular, has been extensively studied for associations with disease. This region is extremely polymorphic with over 103 alleles currently identified (Takeshima et al. 2002). The current genotyping methods, such as PCR-RFLP and direct sequencing, are impractical for screening large populations of animals for the presence of these alleles. Specific allele detection techniques such as PASA, PAMSA, tetra-primer PCR and Bi-PASA can be used to rapidly, and inexpensively, screen large populations for the presence of specific alleles.
A modification of one of these techniques may provide a solution to the problem of screening the \textit{DRB3} gene.