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GENETIC AND EPIGENETIC REGULATION OF THE BOVINE IMMUNE SYSTEM: PRACTICAL IMPLICATIONS OF THE HIGH IMMUNE RESPONSE TECHNOLOGY

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Identifying dairy cows with superior immune response (IR) reduces disease, increases farm profit, improves milk quality and increases animal well-being. In Canada, it costs the dairy producer $110 to $320 per case of mastitis, and it has been estimated that almost 1 out of every 5 dairy quarters in Canada is infected with a mastitis-causing pathogen (Canadian Bovine Mastitis Research Network, *"What's New in the World of Mastitis Research?"* http://www.medvet.umontreal.ca/rcrmb/dynamiques/PDF_AN/Results/NewspaperWhatsNew.pdf. 2009). The emergence of antibiotic resistant pathogens is of increasing concern to the producer, as well as to the public. Around the globe there is a concerted effort to limit the use of antibiotics, particularly in food-producing livestock. Therefore, in keeping with the European Unions' proactive thinking that "prevention is better than cure", alternative methods for disease control are earnestly being sought for animal agriculture. Various genetic approaches are being evaluated as suitable methods to enhance disease resistance of livestock. One of the most attractive options available is to make use of the animal's own immune response genes to select for healthier animals with naturally superior immunity. This approach can work well on both conventional and organic dairy farms. This immunogenetic approach will be the focus of this article.

Genetic Regulation of the Immune System

The immune system is composed of integrated, genetically regulated sets of cells and molecules that control the response to external and internal stimuli, including pathogenic micro-organisms (Delves and Roitt, 2000; Mallard and Wilkie 2007). Improved understanding of the biological and genetic relationships within the immune system during periods of production stress, including following vaccination or disease challenge is helping to facilitate the implementation of new approaches to improve livestock health. Recent studies by our groups and others have focussed on evaluating host defence mechanisms as indicators of specific and broad-based inherent disease resistance. There is clear evidence in rodents, poultry, pigs and cattle that it is possible to selectively breeding for high (H), average (A) or low (L) – immune responsiveness, and that H-responders can positively influence resistance to infectious disease (reviewed by Kelm et al 2001). In most species, including pigs and cattle, heritability (h^2) estimates for antibody and cell-mediated immune responses are sufficient to allow for improvement via genetic selection (reviewed by Mallard 2007; Abdel-Azim et al 2005). In fact, early research by our group showed health and production benefits following genetic identification of cattle and pigs for enhanced IR. This included lower occurrence of mastitis in high immune responders in 2 out of 3 dairy herds tested, as well as improved response to vaccination and colostrum quality

(Wagter et al 2000). More recent research, has substantiated these claims by demonstrating substantial reductions in odds ratio disease scores for mastitis (~4x), ketosis (~3x), metritis (~8x) and retained placenta (~2.5x) of cows with both high antibody and cell-mediated immune responses in a large commercial US herd (DeLaPaz 2008, Table 1). Our group refers to these individuals with both higher and more optimally balanced antibody and cell-mediated immune responses, as High Immune Responders, and we have developed a patented test system to quickly identify these animals within dairy herds. This method is referred to as the *High Immune Response* (HIR) technology.

Using quantitative genetic methods to identify and select individuals with higher breeding values for immune response traits is one of the novel genetic tools that does not require molecular genetic manipulation of the animal and therefore avoids current controversies surrounding production of genetically modified organisms (GMOs). Nonetheless, the advantages of modern molecular genetics techniques are being employed at the laboratory level to identify and study favourable animal genotypes. Genetically selected and immunologically-defined populations possess the variation required to adapt under conditions of natural and artificial selection, and thus can be utilized as a tool to understand the genes and proteins which govern these phenotypes (Glazier et al 2002). For example, as a means to discover new genes or pathways that regulate the immune system of dairy cattle a bovine immune-endocrine microarray chip was produced in our laboratory and has been used to identify genes associated with H, A and L immune responsiveness and to determine genes that affect disease resistance (Tao et al 2007; Nino-Soto et al 2008). Genes identified as being expressed differently between and the H and L responders included immune response transcription factors, cytokines, histocompatibility and T-cell receptor genes (Nino-Soto et al 2008). Additionally, certain bovine IR genes have been shown to express single nucleotide polymorphisms (SNPs) that associate with high and low somatic cell scores and other dairy traits (Sharma et al 2008; Pant et al 2008; Leyva et al 2008a,b). These SNPs may also associate with H and L-IR and are now being examined in this context.

Features that Distinguish High Antibody and High Cell-mediated Immune Response Phenotypes

Practically speaking, since antibody and cellular immunity are key aspects of the adaptive immune system that are critically important in control of extra- and intra-cellular pathogens, respectively, it is best to select animals that have both high antibody and high cell-mediated immune responses. This produces animals with a well balanced IR profile, capable of defending against a wide array of diverse pathogen. The HIR technology is designed to identify those cows and calves with robust and unbiased immune responsiveness that can be kept for future breeding to improve herd health, while low immune responders may be culled from the herd.

Academically, it is of interest to better understand the immunological features that distinguish the various IR phenotypes. In order to do this, we have classified Holsteins ranging in age from 6-30 months as having either high antibody-mediated immune response (HiAMIR) but low cell-mediated; or high cell-mediated immune response (HiCMIR) but with low antibody. As one might have expected those individuals with HiAMIR had significantly more B-lymphocytes which are the cells involved in antibody production compared to HiCMIR animals. Conversely, HiCMIR responders had significantly greater numbers of certain T-lymphocyte subsets,

particularly T-cells of the WC1+ (gamma-delta) phenotype. The T-lymphocytes are those leukocytes particularly important in generating cellular immunity to intra-cellular pathogens. Additionally, T-helper cells support antibody responses to a wide range of T-dependant antigens.

High immune responders identified using the patented test system also had greater numbers of other leukocyte subpopulations. For instance, the HiCMIR animals also had significantly greater numbers of monocytes, whereas the HiAMIR tended to have more neutrophils. Some of these parameters were influenced by age or pregnancy and must be appropriately accounted for in the statistical models (Hine et al 2010). Improved kknowledge of how age and pregnancy influence antibody (type 1) and cell-mediated (type 2) IR will improve our ability to select animals with enhanced immune responsiveness and resistance to various pathogens. It is worth noting that in general a calf identified as a high responder will maintain that classification as a mature lactating cow. Therefore animals only need to be tested and classified based on their IR breeding value once in their lifetime. This information helps us to better understand the mechanisms that underpin the improved immunity of high responders and the increased value of those animals that have both high antibody and cell-mediated immunity.

Evaluating Immune Response in Cohort Herds across Canada

Recently, in collaboration with the Canadian Bovine Mastitis Research Network (CBMRN), 690 cows from 58 herds across Canada were immunized using the patented system to evaluate their IR profiles. Three blood samples and a simple skin test were taken to measure specific antibody and delayed-type hypersensitivity as an *in vivo* indictor of cell-mediated immune responses, respectively. Enhancing both antibody plus cellular immunity is especially important for diseases such as mastitis where there are multiple causative organisms that require various immunological mechanisms to control the disease.

High, average and low immune responders were found within each herd in all regions across Canada. Ranking of cows could be compared within herd, within province and across regions based on either their phenotype or IR breeding values. In this Canadian study approximately 15% of cows were high, 15% were low, and 70% were average immune responders with some slight differences between provinces (Thompson-Crispi et al 2010).

Immune Response and Health

Two other immune response trials have previously demonstrated that HIR cows have the lowest disease occurrence (Wagter et al 2000; DeLaPaz 2008). Now, through the CBMRN, data is available on clinical mastitis cases and analysis is underway to determine the association of HIR on incidence, duration and severity of mastitis. Preliminary results show that among all cases of clinical mastitis in the cows across Canada that were tested for immune response, cows classified as HIR had the lowest occurrence of coagulase-negative staphylococci (CNS).

Immune Response and Production

Results to date have shown that breeding for optimal high immune response based on both antibody and cell-mediated immune responses would not compromise production. There is

evidence that some cows with only HiAMIR have lower milk production but cows with HiCMIR have higher milk production (Wagter et al 2003; DeLaPaz 2008; Mallard 2007). Therefore when both traits are used in a selection index there is no adverse affects on milk yield, fat or protein. Similarly in the CBMRN study there were no differences in 305 day milk yield, protein yield, fat yield or overall lifetime profitability in HIR cows compared to low or average IR cows (Thompson-Crispi et al 2010).

Breeding for Optimal Immune Response in Canadian Holsteins

Heritability is the proportion of the phenotypic variation in a trait that is due to genetics and this information is used to estimate an animal's breeding value for that trait – in other words the ability to transmit those genes to their offspring. Results of the CBMRN study showed the h^2 of the antibody and cell-mediated immune response traits to be moderate to high, ranging from 0.14 – 0.56. This indicates that between 14% and 56% of the phenotypic variation in immune response can be explained by genetic variation. Using these heritability estimates, breeding values were calculated to rank cows for immune responsiveness (Fig 2). These results are similar to those found in previous studies (Mallard et al 2010). Since a significant genetic component has been identified in these IR traits, it is possible to include immune response in breeding programs to make genetic gains in overall dairy health.

Immune Responses to Various Mastitic Strains of *Staphylococcus aureus*

In another aspect of our work, we were interested to evaluate antibody and cell-mediated immune responses to various *S. aureus* strains. In this set of experiments, 4 groups of Holstein cows (5 cows/ treatment) were infected by the intramammary route with one of 4 genetically characterized *S. aureus* strains; the naturally occurring Small Colony Variant (SCV Heba3231) (Atalla et al 2008), its parent strain 3231 (Atalla et al 2008), a genetically defined Newbould *hemB* mutant displaying the SCV phenotype, or the prototype strain Newbould 305.

Infected cows were monitored and given scores for the development of clinical mastitis based on systemic and localized signs (Atalla et al 2009*)*. Both SCV strains induced mild clinical mastitis, while both wild-type strains induced acute clinical mastitis (Atalla et al 2009). Somatic cell scores (SCS) from all treatment groups were significantly ($p < 0.05$) higher at the first 5 days and up to day 36 post-challenge relative to SCS before challenge (Atalla et al 2009).

Anti-*S. aureus* IgG1 and IgG2 responses in sera and whey were determined using sandwich ELISA at days 14, 21 and 36 post-challenge. In addition, cows in each group were inoculated with the UV-killed homologous strain intradermally in the neck at day 24 post-challenge to induce DTH as an indicator of CMIR and differences in % increase double skin fold-thickness were measured at 6 and 24 h (Atalla et al 2010b). Both SCV Heba3231 and its parent strain 3231 induced strong type 1 immune responses as evident by significant ($p < 0.05$) DTH, as well as IgG1 and IgG2 antibody responses in sera and/or milk whey with more of a type 1 bias (Atalla et al 2010). Type 1 immune responses are particularly important in the control of intracellular pathogens, such as SCVs that have the ability to survive inside host cells. Conversely, both the *hemB* mutant and Newbould 305 strains induced a type 2 response as indicated by failure to

develop DTH and predominance of IgG1 antibody response in sera and/or milk whey (Atalla et al 2010).

The expression of cytokine marker genes, TNF-α, IL-8, TGF-β and IL-10, were also determined using real-time PCR in blood-derived mononuclear cells at days 0 before challenge and days 2 and 36 post-challenge. The mRNA of target gene was quantified relative to that of the reference gene β2-microglobulin. In all treatment groups both TNF-α and IL-10 transcripts were differentially expressed following challenge, while IL-8 and TGF-β transcripts were not. Up-regulation of TNF-α during the chronic phase of infection with SCV Heba3231 and the 3231 parent strain seemed to modulate the immune response to allow persistence. Increased IL-10 expression during the chronic late phase of infection to 3231 and Newbould 305 strains was likely to minimize immune-mediated pathology, whereas early expression in response to Newbould 305 seemed to drive the downstream immune response towards a non-protective type 2 response (Atalla and Mallard 2010). Persistence of bovine mastitis appeared linked to the adaptability of *S. aureus* strains to the mammary gland environment. One of the important strategies to overcoming host defences appeared to be the formation of the SCV phenotype.

Finally, we evaluated the possibility of ranking cows as H, A or L immune responders based on *S. aureus* AMIR and CMIR responses. It was evident that these antibody and cell-mediated responses were strain dependent more than cow dependent. Therefore ranking cows based on response to *S. aureus* was not suitable and ranking using the specified type 1 and 2 test antigens as described in the HIR patent remains the best choice for ranking cattle for immune response as a predictor of improved disease resistance.

Epigenetic Regulation of the Immune System

Epigenetic effects in the form of histone modifications and DNA methylation are actively involved in the induction, stability and clonal inheritance of gene expression in T-lymphocytes, particularly T-helper 1 and 2 cells as they impact disease resistance (Wilson et al, 2009). Specifically, DNA methylation appears to have modulating effects on the interferon-gamma (*IFNG)* and interleukin (*IL)-4* promoters of humans, as well as several other species, including dairy cattle (Sanderse et al, 2006; Schoenburn et al, 2006; Paibomesai et al 2010). This cytokine expression is important for the induction of T-helper 1 and 2 subtype responses that can strongly influence type 1 and 2 immune responses. In fact, epigenetics is now generally thought to represent a vital connection between gene expression and the environment (Petronis, 2010). As such, a better understanding of DNA methylation, as well as other epigenetic modifications, serves as a critical component to links molecular, cellular and physiological responses, including immune responses that control disease resistance.

To determine whether epigenetic effects may be playing a role in the shift in type 1 and type 2 immune response bias that occurs during pregnancy and parturition of dairy cows we examined DNA methylation of bovine type 1 (IFN-γ) and type 2 (IL-4) cytokine promoter genes between wk -4 and day 4 relative to calving. The T-cell mitogen, ConA, was used to stimulate CD4+ T-helper cells to proliferate and induce cytokine production. Using ELISA to evaluate cytokine production an increase in both cytokines was observed following mitogen treatment. DNA

methylation of genomic DNA was evaluated using a bisulphite DNA methylation kit. A general decrease in methylation in the *IFNG* promoter (-8.8%) was noted which was consistent with the noted increase in IFN- γ production. Conversely, ConA stimulation was associated with a general increase in methylation at the *IL4* promoter (+13.9%) (Hussey et al 2010). Inverse methylation patterns between these two cytokines have been previously reported for other species and are consistent with their opposing regulatory functions (Wilson et al 2009).

The synthetic glucocorticoid, dexamethasone (Dex), was then used to simulate corticosteroid effects that can occur around parturition. Epigenetic influences in the 2 cytokine promoters in response to this immunosuppressive hormone indicated increased methylation of IFNG (+18%) and decreased methylation of IL4 (-31%) (Hussey et al 2010). Sub-optimal IR, increases disease risk, and changes in the balance between type 1 and type 2 IR during peripartum have been previously reported (Shaver-Weaver et al 1999; Wagter et al 2000; Sordillo et al 2009). However, epigenetic influences on bovine cytokine genes, known to steer type 1 and 2 IR, have not been previously reported and as seen here are expected to play a critical role in the IR bias that dictates the nature of immunity during the calving period.

Practical Implication of the High Immune Response (HIR) Technology

Breeding companies distribute sire proofs (breeding values) to improve mastitis that include Somatic Cell Score (SCS) as an indicator of udder health. SCS, however focuses only on one disease, whereas HIR focuses on broad-based disease resistance. Breeding companies in Canada, including the Semex Alliance, are also beginning to distribute semen from various breeds that are more resistant to disease; for example, Norwegian Red cattle. Dairy Herd Improvement (DHI) companies, such as CanWest DHI provide information on SCS and bacterial colony forming units in individual milk samples, as well as offer diagnostic milk ELISA tests for *S. aureus* mastitis, Johne's Disease, and Bovine Leukosis Virus. Nutrition companies are offering rations that support optimal health and may enhance immune response while pharmaceutical companies market and distribute vaccines that prevent respiratory and gastrointestinal infections in cattle, and Gram negative intramammary infections in lactating cows. However, no company as of yet has attempted to produce a product or service that provides the producer or breeding company with an indication of how well their cattle may respond and/or resist infection to many different micro-organisms, and HIR technology is designed to meet that need. Further this technology offers a solution that results in a reduction in disease occurrence, a reduced use of antibiotics, a reduction in the cost of food animal production, and this translates into an increased quality of food for the consumer.

Qualitative market research was conducted by an independent firm, Agri-Studies (Guelph, Ontario), using 3 focus groups to assess interest in the HIR technology among dairy producers and the dairy support industry, including pharmaceutical companies, dairy herd improvement organizations, veterinarians, breeding and feed companies, and government. Results showed significant interest among dairy producers to use HIR to identify calves or cows with High Immune Response (75% of producers). They acknowledged that the technology would provide beneficial information for culling decisions, grouping, breeding, and/or treating animals, but the key benefit they saw was the ability to cull animals as calves and save the cost of raising animal that later may have significant health issues. They also saw the value of using sires that were classified as HIR to improve the health of their herds. Among participants from the dairy support

industry, the most common benefits cited included the use of HIR technology as a diagnostic tool to target therapeutic drugs or vaccines toward the various IR phenotypes, to improve genetics, and to increase business opportunities with dairy producers. Further market assessment and beta testing of dairy herds is now underway to finalize the transferability of the technology to the marketplace.

HIGH IMMUNE RESPONSE (HIR) ANIMALS ARE NATURALLY IMMUNE

HIR is a patented evaluation technology developed to identify dairy cattle with high adaptive immune response capability.

Identification is safe, fast and effective.

Benefits include:

- Lower disease occurrence and severity
- Reduced treatment and veterinary costs
- Increased response to vaccines
- Increased colostrum quality
- Cows as young as 2 months can be tested
- Animals only need to be tested once in a lifetime
- Testing is safe and does not interfere with any other diagnostic testing
- Cost benefit analysis show significant savings to producers who identify HIR cows in their herd.

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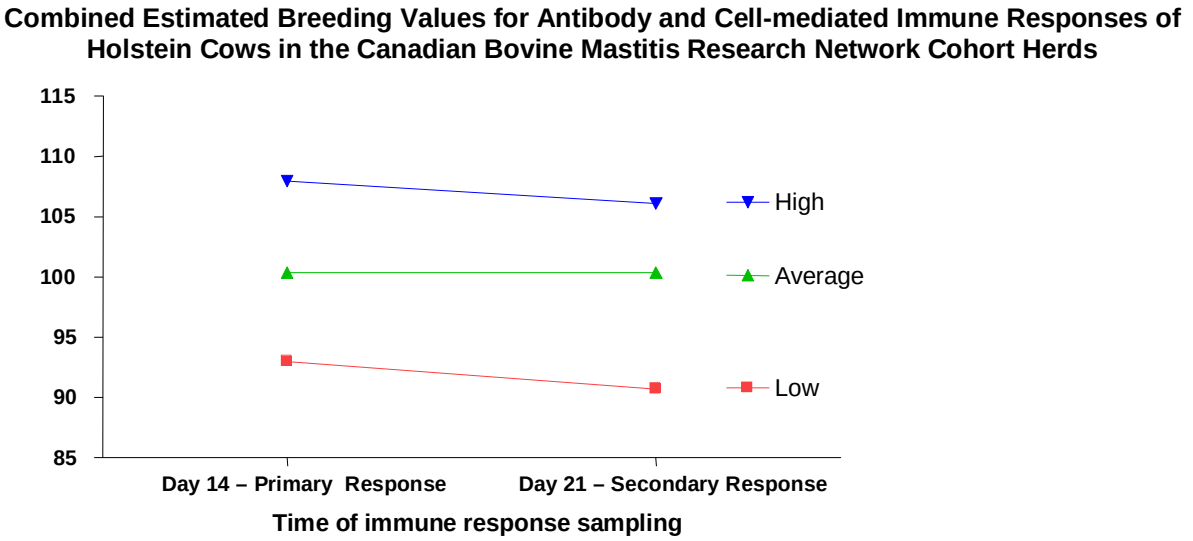
Table 1. Estimated Odds Ratio (and Confidence Intervals) of Disease Incidence by Immune Category in Peripartum Holstein Cows (n=875) from Large US Dairy Herd.

Disease	Mastitis		Metritis		Ketosis		Retained Placenta	
Immune Response Category	AMIR	CMIR	AMIR	CMIR	AMIR	CMIR	AMIR	CMIR
High versus Average Immune Responders	1.76	2.14	0.52	7.40	1.75	1.05	0.65	1.94
Confidence Interval	(1.08-4.08)		(0.23-60.25)		(0.40-4.45)		(0.21-6.45)	
AMIR + CMIR Odds Ratios for High vs Average Responders	3.9		7.9		2.8		2.6	
Disease Occurrence During this Study	22% Mastitis		5.3% Metritis		5.8% Ketosis		7.4% Retained Placenta	

Source: Adapted from - DeLaPaz, Jason. MSc Thesis, University of Florida, 2008.
AMIR, Antibody-mediated Immune Response; CMIR, Cell-mediated Immune Response.


Combined Estimated Breeding Values for Antibody and Cell-mediated Immune Responses of Holstein Cows in the Canadian Bovine Mastitis Research Network Cohort Herds
115
110
105
100
95
90
85
High
Average
Low
Day 14 – Primary Response
Day 21 – Secondary Response
Time of immune response sampling