The Genetics of Bovine Vaccination

Richard Jonathan Leach

Department of Genetics and Genomics
The Roslin Institute
The University of Edinburgh

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Declaration of Originality

I herby declare that the research described in this thesis and the thesis itself was composed and originated entirely by myself, except where otherwise stated.

Richard Leach

February 2011

List of Publications

Peer Reviewed Publications

The Bovine Genome Sequencing and Analysis Consortium., Elsik, C. G., Tellam, R. L., Worley, K. C., *The Genome Sequence of Taurine Cattle: A Window to Ruminant Biology and Evolution.* Science, 2009. **324**(5926): p. 522-528.

Glass, E. J., Baxter, R., **Leach, R. J**., Jann, O.C. *Genes controlling vaccine responses and disease resistance to respiratory viral pathogens in cattle*. Veterinary Immunology and Immunopathology. (In press)

Leach, R. J., Craigmile, S.C., Knott, S. A., Glass, E. J. *Quantitative trait loci for variation in immune response to a Foot-and-Mouth disease peptide.* BMC Genet, 2010. **11**(1): 107 (Based on chapter 2 in this thesis).

Leach, R. J., Craigmile, S.C., Knott, S. A., Glass, E. J. *Quantitative trait loci for variation in immune response to a Bovine Respiratory Syncytial Virus (BRSV) vaccine*. Vaccine (Submitted) (Based on chapter 3 in this thesis).

Conference Papers

Hadjipavlou, G., Hemani, G., **Leach, R**., Louro, B., Nadaf, J., Rowe, S., de Koning, D. J. *Extensive QTL and Association analyses of the QTLMAS2009 data.* BMC proceedings, 2010. **4**(Suppl 1)**:**S11.

Leach, R. J., Craigmile, S.C., Jann, O.C., Glass, E. J. *Locating SNPs in the bovine genome associated with variation in the immune response.* The 9th World Congress on Genetics Applied to Livestock Production, 2010. Paper number: 486.

Book Chapter

Glass, E. J., Baxter, R., **Leach, R**. and Taylor, G. Bovine viral diseases. *The role of host genetics*. Chapter in "Breeding for Disease Resistance in Farm Animals" pages 88-141. 3rd edition (2010). Edited by S. C. Bishop., R. F. E. Axford., J. B. Owen and F. W. Nicholas, CAB International, Nosworthy Way, Wallingford OX10 8DE, United Kingdom.

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*"… I'd also like to thank my local **Abbot** for his mind expanding thoughts…"*

Abstract

Infectious disease is an important issue for animal breeders, farmers and governments. Solutions to control infectious disease are needed and research focused on the genetic loci determining variation in immune-related traits has the potential to deliver solutions. The primary aim of this thesis is to discover regions of the bovine genome which influence the immune response post immunisation. To accomplish this two types of immunising agents, a Foot-and-Mouth Disease Virus (FMDV) peptide (FMDV15) and a commercial vaccine for Bovine Respiratory Syncytial Virus (BRSV), were used to immunise the second generation (F2 and backcrosses) of the Roslin Bovine Genome (RoBoGen) herd, a Charolais Holstein cross population. The FMDV15 peptide consisted of two sections of the VP1 protein located on the FMDV capsid, together encompassing the major neutralising antibody sites that are known to be immunogenic. Protection against FMDV is generally believed to relate to the levels of neutralising antibody and has been correlated with IgG1 and IgG2 levels as well as interferon-γ. In addition it has been shown that T cell responses also play a role in protection against FMDV. Thus all of these were used as phenotypic measurements post immunisation to the FMDV15 peptide. The BRSV vaccine used was an attenuated live vaccine. Protective mechanisms against BRSV infection include IgA, IgG1, IgG2 and IgM BRSV-specific antibodies and antibody titres particularly those of the IgG isotypes are considered to be correlates of protection. Thus, IgG1 and IgG2 antibody levels were measured post vaccination with the BRSV vaccine. All phenotypes were measured across time, and allowed analysis of the primary and secondary adaptive immune responses.

Both agents caused considerable variation in the phenotypes measured post immunisation, with significant responses detected two weeks post immunisation. REstricted Maximum Likelihood (REML) analysis attributed much of this variation to sire, highlighting the heritable component, and environmental effects. Significant positive correlations were detected across time within each trait for both the FMDV and BRSV responses. The FMDV and BRSV antibody levels also correlated with

each other at later time points, suggesting that there may be animals which are genetically predisposed to be high or low responders in general. Initially a linkage mappingapproach was followed using 165 microsatellite markers, which detected 77 QTL in response to the FMDV peptide and 27 QTL in response to the BRSV vaccine. There were some overlapping QTL, for example QTL which spanned the Major Histocompatibility Complex. Further analysis was conducted by developing a Perl scripted program which genotyped the RoBoGen herd in two ways; 1) Single Nucleotide Polymorphism(s) (SNP) were genotyped within the confidence intervals of the previously discovered QTL and 2) SNP were genotyped via a candidate gene approach. Association study methodology, accounting for relationship stratification via principal components of the genetic relationship matrix, was used to detect significant SNP, in response to both the FMDV peptide and the BRSV vaccine. Twenty significant SNP associations were discovered across 19 traits, with some SNP located in genes with known biological relevance to an immune response, such as the Toll-Like Receptors (TLR), TLR4 and TLR8.

This thesis has detected regions of the genome which are significantly associated with the immune responses elicited by two different agents, suggesting similar pathway(s)/gene(s) may be used in defence of multiple pathogens. Once regions of significance were detected, further analysis using SNP markers identified significant, non-synonymous SNP that were associated with the immunising agents. The novel markers discovered in this study may aid breeding for resistance to disease via marker assisted selection. In addition, they may also have highlighted new targets for vaccinologists to develop ‘next generation’ vaccines.

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General Introduction

1.1 Introduction

Infectious disease of livestock continues to be a cause of substantial economic loss and has adverse welfare consequences, even in well managed agricultural systems [1]. In addition, even with stringent bio-security, there are incursions of "exotic" diseases e.g. the recent Foot-and-Mouth Disease (FMD) outbreaks within the E.U. [2]. Current interventions against infectious disease include anthelminthics, antibiotics and other chemicals as well as vaccination, although for many endemic and exotic diseases there are limited appropriate and effective controls. Thus alternative solutions for disease control are needed. Breeding for disease resistance together with more effective vaccines have the potential to deliver solutions.

There is considerable variation among individuals in the response to infectious disease and vaccination, a significant proportion of which can be shown to be genetic [1]. It is clear that the wide range in immune responsiveness and disease resistance found within livestock population is controlled by many genes [3]. Many candidates genes have been identified that may influence the immune response, including the Major Histocompatibility Complex (MHC), however, the relative contribution of the MHC and non-MHC loci to the wide variation in immune-related traits is only beginning to be explored. Identifying and understanding the role of different polymorphic loci in immune-related traits may lead to the identification of selectable markers for disease resistance, and may also suggest new host targets to improve vaccine efficacy.

Identifying the causal genes involved in disease resistance and vaccine response is not straightforward. Most studies in livestock mammals have used field data, with the inherent problem of variation caused by considerable environmental influences, and where the phenotypes are generally based on morbidity and mortality as determined by veterinary observation. An example of veterinary observation is shown by *Casas et al* [4], where a veterinarian defined the presence bovine keratoconjunctivitis infection as a phenotype. These forms of classification fail to

realise the whole spectrum of the immune response, which can also be viewed as a quantitative response. Thus much of the variance from the immune response is ignored, making statistically significant associations with fixed or random effects more difficult to detect. However, the actual correlates of protection are often unknown and likely to be a complex combination of innate and acquired immunity. Many diseases, such as mastitis can be caused by distinct pathogens which result in different responses that are likely to be under different genetic controls [5]. The causal pathogen is often not identified in field studies. In addition, field data consists of single time points and cannot account for variation in the kinetics of immune responses.

1.1.1 Genetics of Vaccination

Genetic variation has not been explored thoroughly in terms of response to vaccination [6], even in humans [7]. It is clear that there is variability in host response to vaccination, but many factors account for this. In order to concentrate on the genetic factors involved in the immune response post immunisation, most studies have attempted to reduce or separate out the other factors which affect the phenotypic traits. In practice this either means reducing environmental variables as much as possible and/or conducting large scale studies in a population with a complete pedigree.

Although this project is focused on cattle, it is important to take account of other research to gain a full insight of the current issues in the field of vaccination, in particular the genetics behind vaccination. Initially most studies investigated the role of the highly polymorphic MHC region, due to its known role during an immune response. However it is becoming clear that non MHC genes control a large proportion of the genetic variability in immune responses to vaccination [6, 8-9].

In the human field, typical examples of variation in the immune response post vaccination include responses to measles and hepatitis B. Measles is still causing significant world health problems for the young. Currently there is a two dose vaccine which maintains protection from measles in vaccinated populations but there

is variability in the response. Non responders to the vaccination have been associated with dysfunction of T_h1 and specific B cells [10-11], which indicates the likely polygenic nature of the underlying genetic causes. These results also highlight that protection relies on several immune pathways, thus a vaccine which effectively induces protection by stimulating appropriate immune pathways is needed. One method to make vaccines more efficacious is to understand the genetics of responder individuals. Discovering the genetics associated with the response to measles vaccination has begun [7, 12-15], and it is apparent that responders to the measles vaccine appear to lack the 'non responder' alleles rather than having specific responder alleles [12]. In addition, the recently available recombinant vaccine against hepatitis B is also an example of a vaccine resulting in variation in response, as 5-10% of people vaccinated are complete non responders [16]. The research compiled on hepatitis B vaccination and infection to date shows similar conclusions to that of the research on measles vaccination; there is a large genetic component to the response to infection or vaccination with hepatitis B [8, 10, 16-20].

Genome wide association studies in humans are also providing targets for treatment of infectious diseases. Hepatitis C virus (HCV), for example, infects 170 million people a year (http://www.who.int/), and infection carries the risk of liver fibrosis and subsequent cirrhosis and hepatocellular carcinoma [21]. Recently, genome wide association studies associated HCV infection with IL28B [22-24]. This finding has led to the implementation of clinical trials [25], potentially discovering a novel treatment for HCV infection. This is an encouraging example of the potential of what whole genome scans could contribute to the cattle industry, possibly improving vaccine efficacy by detecting pivotal genes. One possibility for improving vaccines is the potential to develop analogues of pivotal gene products. Thus, if a non responder allele confers poor production of protein, these individuals could have vaccines tailored to include the protein analogues.

Vaccination of cattle also elicits a highly variable immune response [6], which indicates that a genetic component plays a role in the variation. However, studies investigating the variation post vaccination often do not investigate the genetic loci

associated with the variation (examples include, [26-27]). Studies investigating post infection phenotypes have shown associations with genetic loci that account for a proportion of the variability in the immune response (section 1.2.2), strongly suggesting there is also a genetic component involved in the immune response post vaccination.

1.2 Relevant Phenotypes

1.2.1 The Immune system

To detect regions of the bovine genome which influence the immune response following immunisation, a detailed phenotype is required which is able to capture the variation of the immune response in a herd. In addition, as the immune response is a qualitative response which constantly changes, measuring the response over several time points, will allow analysis of how the immune response reacts to an immunogen. However, an understanding of the immune response is required to collect appropriate immune related phenotypes and measure the phenotypes at relevant time points.

The immune system consists of highly complex components that detect and defend against foreign bodies (pathogens) from infecting the host [28] and causing disease (However, in certain cases the immune response itself can also cause disease [28]). To fight infection the immune system orchestrates innate and adaptive immune divisions. The innate immune system is the first line of host defence [29] and reacts to a wide range of pathogens [30]. If the innate immune system fails to eliminate a pathogen then vertebrates have a secondary defence, the adaptive immune response [31-32]. Once alerted to a pathogen via the innate immune response the adaptive response is antigen specific and involves the generation of immunological memory (Figure 1.1) [33].

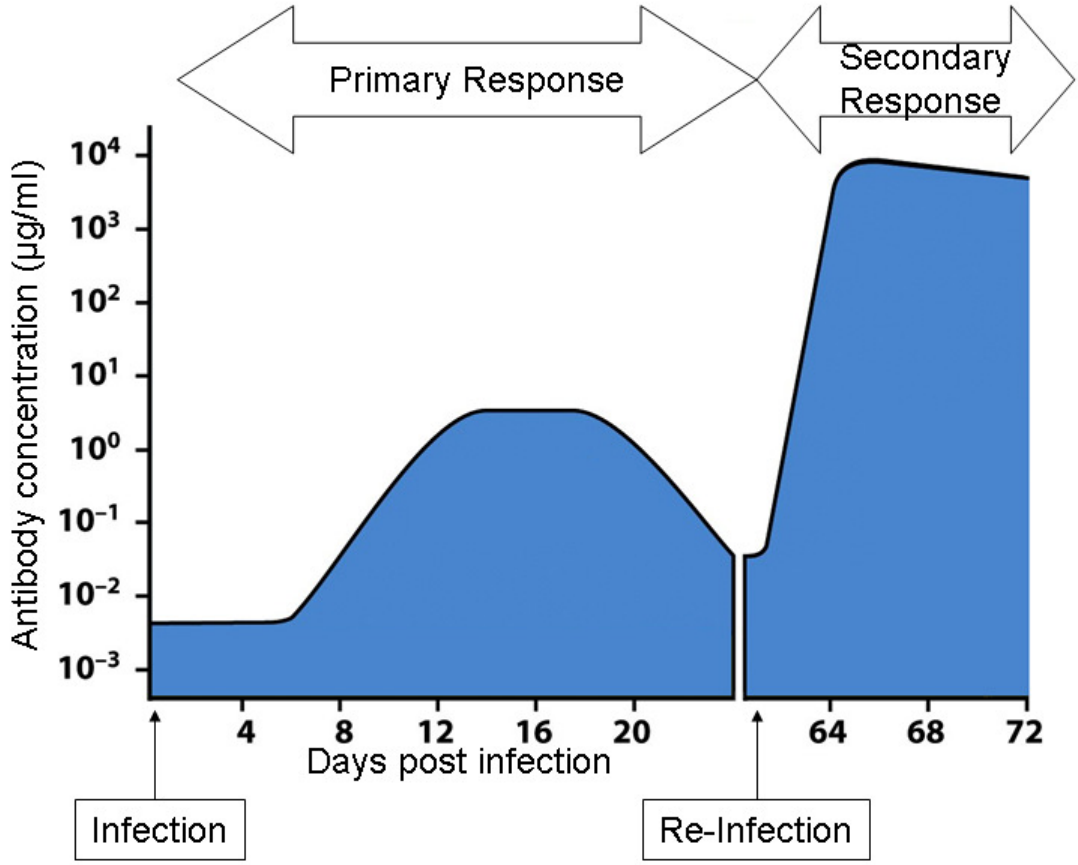


Figure 1.1: An adaptive immune response. When the adaptive immune system initially encounters an antigen, the primary response is elicited after a lag phase. If the adaptive immune system encounters the antigen again a swift and stronger secondary response is elicited. Based on an illustration within *Janeway et al* [10].

1.2.1.1 Innate immunity

As the innate immune system communicates and directs with the adaptive immune system [34], which ultimately confers immunity [33], the genes and pathways which the innate immune response uses are of great interest to vaccinologists and animal breeders as they may enable a higher percentage of animals to elicit appropriate responses to disease. Understanding the mechanisms which elicit an adaptive immune response may also explain some of the variance detected in the immune response post vaccination.

Innate immunity is one of the first barriers to infection. It detects pathogens in many different ways, for example: using Pathogen Recognition Receptors (PRR) [30, 35],

such as Toll-Like Receptors (TLRs) [30] and Nod Like Receptors (NLR) [35] which are located on Antigen Presenting Cells (APC) such as Macrophages (MΦ) and Dendritic Cells (DC) [28]. PRR are coded into the germline and are able to detect conserved regions of bacteria, viruses and fungi [30], called Pathogen-Associated Molecular Patterns (PAMP). Once a PRR is stimulated by a PAMP multiple distinct signalling pathways become activated. Both immunogens investigated in this thesis are derived from viral pathogens. In the case of viral infection, key molecules are transcription factors such as Interferon Regulatory Factor (IRF) 3, 7 and Nuclear Factor κB (NF- κB) [36]. These transcription factors cause the secretion of cytokines such as interferons (IFN) [36], which are critical in coordinating the innate and adaptive responses [34] against viral infection. Thus variations in the innate immune response can vastly affect how it communicates with, and elicits, the adaptive immune response [34].

1.2.1.2 Adaptive immunity

The adaptive immune response is initiated within the lymphatic organs, which have evolved to provide an ideal environment for DCs to present antigen to naïve B cells and T cells [34]. During a virus elicited response, type II IFN and other cytokines such as interleukins 12 and 18 produced by the innate immune response cause $CD4^+$ T cells to polarize into Th1 cells [37]. Indeed, IFN-γ, a type II IFN mainly produced by Natural Killer (NK) cells [38], has been shown to play a key role during viral infections [36] and has been used as a phenotype to measure the immune response to different pathogens [39-40]. In addition, during activation of differing subsets of T cells [41], $CD8^+$ T cells will differentiate to Cytotoxic T Cells (CTL), enabling them to kill virus infected cells [41], via communication with Major Histocompatibility Complex (MHC) class I molecules [42]. Further, activation of B cells, during a viral infection means that antibody production can be elicited [34]. Thus, antigen specific antibody levels and T cell levels can be used as phenotypes to follow the adaptive immune response.

The MHC is an essential link between an innate and adaptive immune response as it used to communicate between T cells and antigen presenting cells (APC). In cattle

the MHC region is named the Bovine Leukocyte Antigen (BoLA) and consists of three classes: I, II and III [43]. The MHC class I molecules bind to peptides derived from a cell's expressed proteins and presents them on the cell surface [44]. Thus if the cell is infected by a virus, peptides from the virus will be expressed on the cell surface and be detected by CTL [41]. Class I MHC has been shown to play a role in the clearance of viral infections in cattle, for example, against foot and mouth disease virus (FMDV) [45]. MHC class II molecules bind peptides which have originated from pathogens that have been phagocytised by APC, such as MΦ and DC, and are expressed on their cell surface [34]. Class II MHC polymorphisms have been associated with variation in antibody responses and cell mediated responses to a number of viral infections such as bovine leukaemia virus [46] and FMDV [47]. MHC class III molecules encode many gene products with functions from cell cytotoxicity to enzymatic activity [43, 48]. The MHC genes are highly polymorphic to enable it to bind to many pathogen types. However, genetic variations from animal to animal may mean that some processed peptides may not bind or only bind at low affinity [6]. Thus variations in the MHC may also have large impacts on an immune response.

Once a virus has been eliminated or become latent [49] within the host, specific T cell and B cell populations will significantly decrease [34]. However, T memory and B memory cells remain to protect against re-challenge with the same or an antigenically related pathogen [33].

1.2.2 Immunity and resistance

The adaptive immune response is very important in generating immunity within animals, thus both cell mediated and humoral responses are measured as phenotypes in cattle to quantify the immune response to many pathogen types, examples include fungal [50], parasitic [26], bacterial [51] and viral [52] pathogens. Antibody levels, which is the simplest measure of an adaptive response to a pathogen [3], can be correlated with protection [53]. A proportion of the variation in the immune responses across herds [51-52], appears to be genetic, with breed and sire effects

detected [27, 54-55]. As a heritable genetic component exists, breeding animals for a more disease resistant herd is possible [56].

Cell mediated and humoral immune responses have been also been measured post vaccination in cattle, as phenotypes and attributed a proportion of the observed variation to genetics [26-27, 57]. Detecting and locating the genes underlying this genetic component has the potential to turn non or low responders to vaccination into responders. By targeting the genes causing the high/low response, it may be possible to improve vaccine components to better target the low-responders.

Many genes, pathways and physical barriers make up the immune system. In chapters 2, 3 and 4 of this thesis, regions of the genome are shown to be significantly associated with the immune response post immunisation. Few of these regions have known immune function, thus only the major genes and pathways of the immune response have been studied, and where appropriate, more detailed explanations of the immune response will follow in later chapters.

1.3 Genotypes

1.3.1 The Bovine Genome Sequence

The bovine genome is an excellent resource and can be used in many ways. For example, finding a marker that is associated to the immune response is the basis of Marker Assisted Selection (section 1.3.4). Further, placing a significant marker into the genome to discover where it is positioned is a different process involving bioinformatics. Once a marker is placed, further analysis of the biological context is possible, for example, the marker may exist within a gene of known immunological relevance, highlighting possible pathways which have an effect on a phenotype. However a highly detailed and annotated genome sequence is required.

The bovine genome (http://www.hgsc.bcm.tmc.edu/projects/bovine/) contains 30 chromosomes (2n=60, XY). In recent years, sequencing of the bovine genome has

increased rapidly [58]. The initial genetic map of the bovine genome in 1986 [59], had enough resolution to detect synteny between species and their genetic locations within the genome. The resolution of this map steadily increased [60-61] and expanded [62-63] throughout the 1990's. The next big jump came in the form of radiation hybrid maps which provided much more detail of the genome [64]. The current bovine genome sequence was released in 2007 with a 7.1 times coverage of the *Bos taurus* genome. For assembly of the sequenced genome in 2007, two methods were utilised. The first method used whole genome shotgun (WGS) sequencing, following the example of the rat genome construction [65]. The other method followed the human genome approach [66], which used Bacterial Artificial Chromosomes (BAC) for construction [67].

Following the sequencing, a large international effort was made to annotate the genome [68]. This was led by National Institutes of Health (NIH) and the United States Department of Agriculture (USDA), and was successful in bringing the international scientific community together to annotate all aspects of the genome, of which, I contributed to the annotation of immune related genes [68]. Although the annotation effort was a great achievement in itself much work is still needed to confirm the ordering of some scaffolds and to further annotate the genome to a higher standard, as many genes within the bovine genome are still currently either un-annotated or annotated predictions (computer annotations), with no known gene functions (http://www.ensembl.org).

1.3.2 Detection of Quantitative Trait Loci

A Quantitative Trait Locus (QTL) is a region of the genome which is significantly associated with phenotypic variation. To detect QTL, a region of the genome must contain a genetic marker that is either in Linkage Disequilibrium (LD) with a causal polymorphism, or be the causal polymorphism [69]. During the early 1980s the development of molecular technologies to genotype markers began [70], which has led to the production of panels with hundreds of thousands of markers upon them (e.g. http://www.affymetrix.com), greatly increasing the chance of discovering QTL. However it was *Lander and Botstein* [71] who revolutionised QTL detection via

interval mapping using a maximum likelihood (ML) approach to map QTL lying within a chromosome region surrounded by two markers. With the constant addition of more markers though, these methods became computationally intensive. *Haley and Knott* [72] addressed this issue using a regression approach, which approximated the interval mapping method, thus needing far less computational demand. Following this, refinements were made [73-74], as well as variations in methodology, with: ML [71], regression [72] and Bayesian [75] methodologies all being implemented. However, there is little difference between the final results of ML and regression [76].

The two methods for QTL detection used in this thesis are Linkage Mapping (LM), implementing the Haley and Knott [72] methodology which uses a general linear model to detect QTL and Association Study (AS) methodology using REsidual Maximum Likelihood (REML) [77], using the package nlme within R (http://127.0.0.1:14665/library/nlme/html/nlme.html). Both methods of QTL detection rely on the co-inheritance of adjacent DNA variants, with linkage mapping capitalizing on this by identifying haplotypes that are inherited intact over several generations, and the association study methodology relying on the retention of adjacent DNA variants over many generations [78]. However, differences occur in the principles used by these methods to detect QTL.

1.3.2.1 Linkage Mapping

Linkage mapping [72], tracks within-family segregation of linked markers, thus it focuses on recent ancestry, in which there have been relatively few opportunities for recombination to occur. As a result disease associated regions of the genome that are identified by linkage mapping will often be large, and can encompass hundreds of possible genes across many megabases of DNA [79].

To detect QTL, LM uses pedigree information in combination with a linkage map and the genotypes of the animals to infer the Identity By Descent (IBD) of chromosome segments for each animal at specified distances along the genome [80]. During this thesis the genotype probability of each locus at 1 centiMorgan (cM)

intervals on each chromosome was calculated. For each locus position a general linear model is fitted against the phenotype [81], using an F-Statistic as a measure of significance. Figure 1.2 shows a hypothetical QTL calculated using 4 markers.

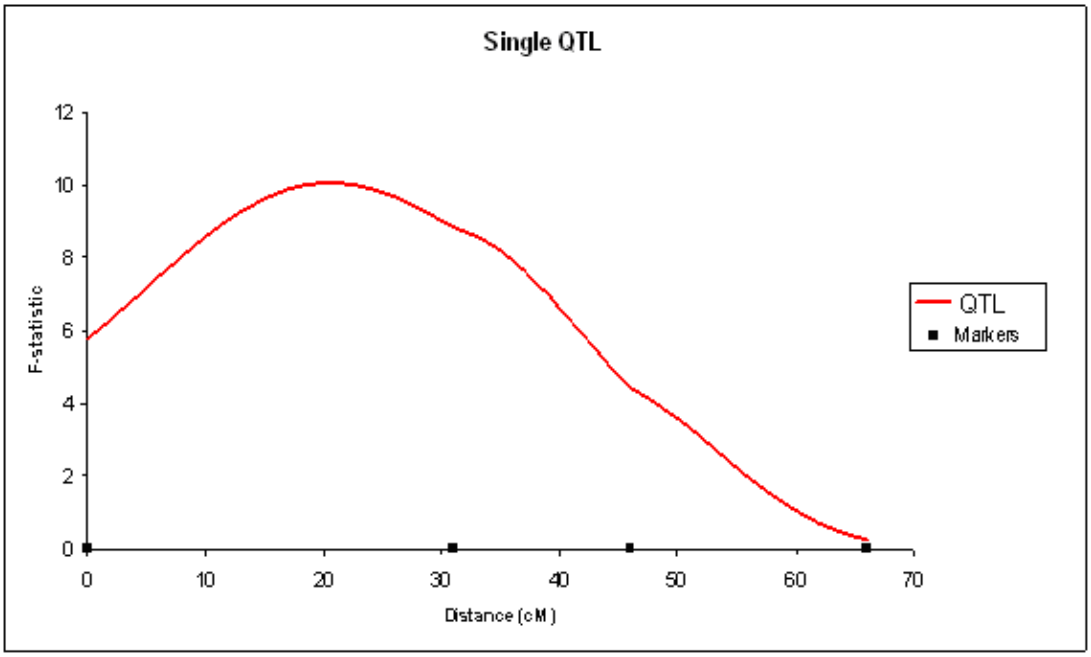


Figure 1.2. The graph depicts a hypothetic QTL. The X-axis is the length along a chromosome; the black squares along the X-axis are the locations of markers on the chromosome. The Y-axis is the measure of significance (F-Statistic). The plotted red line shows the significance of the QTL at each point of the chromosome. In this case the QTL is most significant at 21cM.

LM is credited as a good technique to detect the correct position of a QTL [69], In addition, due to the use of within-family LD, LM studies are better suited to detect low penetrance genes, compared with an association study [82]. However, as the RoBoGen herd was not very large and had few founders, few recombination events will have occurred in the herd, thus large haplotype blocks will be present, making precise detection of QTL difficult using LM methodology. This will result in large confidence intervals for QTL. The confidence intervals of QTL also reflect the QTL effect size, and the density of the markers used [83].

1.3.2.2 Association Study

The second approach used to detect QTL in this thesis is Association. The basic principle underlying the identification of QTL using AS methodology is simple: every animal phenotyped is also genotyped. If a difference exists in the mean

phenotype among the different genotype classes of a marker, then that marker is in LD with a QTL.

In small populations, the amount of LD between tightly linked markers generated by random drift or admixture may be sufficient to allow fine mapping of major genes using a random population sample [69]. As a result, AS relies upon a QTL being in population-wide LD with a casual mutation. As this approach relies on LD at the *population* level, rather than at the *family* level which LM relies upon, it does not track within family segregation, and thus it has benefits in unstructured populations or populations with unknown pedigrees as only one generation needs to be genotyped.

For this approach I used a REML model to calculate the significance of marker effects, as it is able to simultaneously account for multiple factors affecting the trait. The REML model associated each marker, one by one, with a phenotype. Single Nucleotide Polymorphisms (SNPs) are generally used as markers in AS [84] as they are plentiful across the genome (thus specific SNP can be chosen for candidate gene studies, or whole panels of SNPs can be used in GWAS) and relatively cheap [85]. SNP have 3 possible genotypes, which the REML model uses to estimate the effect for each marker in a study (Figure 1.3).

As a pedigree does exist in the current study it was accounted for within the REML model using a genetic relationship matrix. Both the AS and the LM methods will provide similar estimates of QTL effect [86-87].Further, both methods (LM and AS) allow the addition of covariates (all covariates are linear in the current study) and fixed effects to obtain the most appropriate model to detect QTL. Thus highly similar models were used in both approaches. Furthermore both models are able to detect 2 QTL upon the same chromosome.

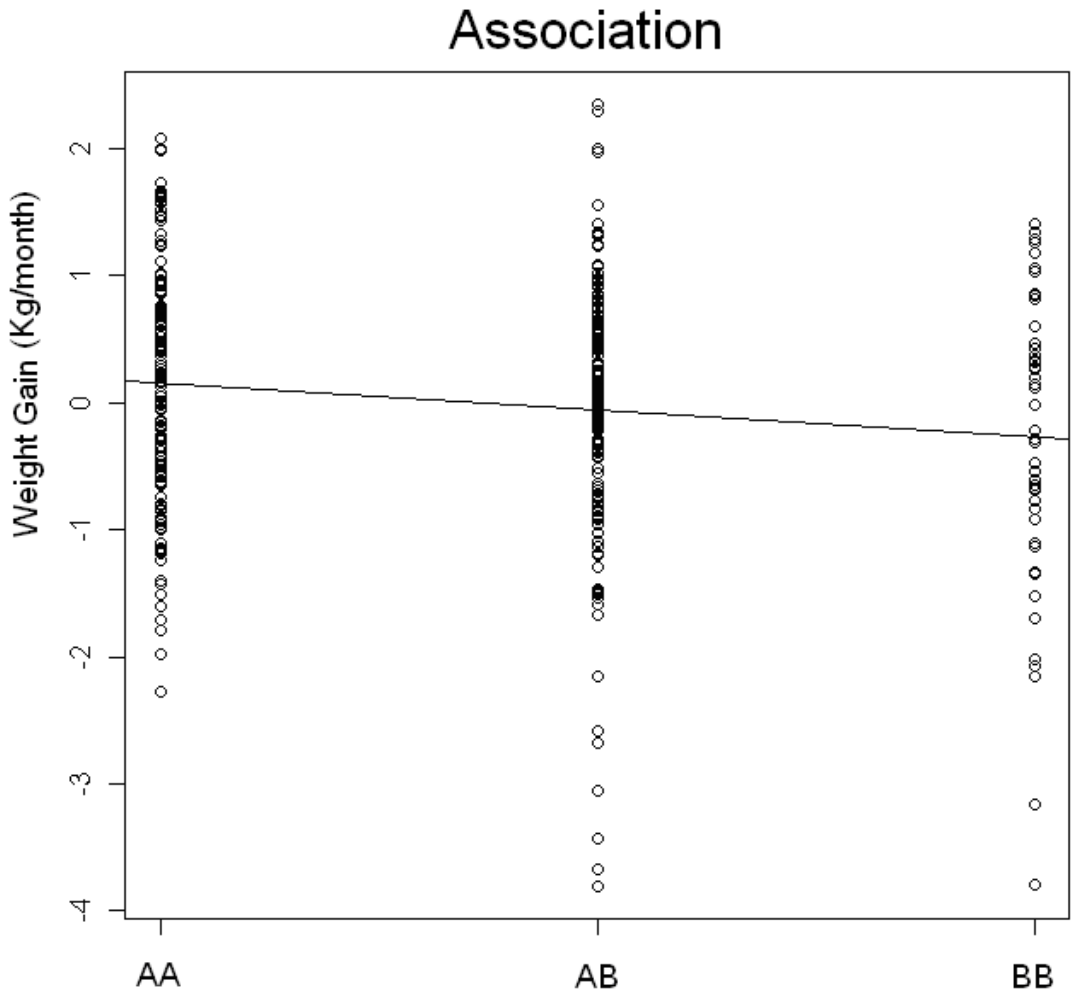


Figure 1.3 The three genotypes of a hypothetical marker are represented along the X-axis. The phenotype (weight gain) is shown on the Y-axis. The regressed line in the graph shows the effect of this marker (assuming an additive model). Thus the animals with the A allele weigh more than those with a B allele.

Both forms of QTL (LM and AS) detection have the potential to improve when extra markers are added. However, as the number of markers increase, more statistical tests are made, and these need to be accounted for in the analysis [88] (this is discussed further in section 1.3.3.).

Association studies are well suited to detect large and medium genetic effects. In addition, due to the expansion of SNP chip technology and the decrease in the price of genotyping [85], AS have become a common and successful method of QTL

detection. However, much of the heritability is left unexplained during the analysis complex traits [89]. Below I have highlighted some of the technical issues that cause AS to miss, or simply not detect, heritable genetic variants that may be associated with complex traits:

Alleles with small effect size

A strength of AS is that it can test many thousands of SNPs across a genome for a significant association with a trait. However, this is also a drawback. As many tests are completed the true causal variants in LD with a SNP are 'swamped' with statistical noise from the other markers that *aren't* associated with a complex trait, causing positive findings to be disregarded as insignificant. This is discussed further in section 1.3.3. It has been recently suggested that much of the missing heritability in AS is due to marker effects being too small to pass stringent significance tests [90].

SNP selection

SNP chip designs have been optimised to capture the maximum amount of genetic variation in a genome. In addition the assumption of 'common disease common variant' (CDCV) [91], argues that common variants may underlie many common diseases, and that common variants would be more easily found using population-based LD rather than family-based LD (such as LM), even if this required testing every gene in the genome. Thus only high-frequency variants that capture the largest proportion of genetic variation are included in the majority of studies and commercially available SNP chips. However, rare variants are thought to, at least contribute, to complex traits [92]. Thus by not including rare variants in an investigation, phenotypic variance and heritability that could be attributed to the rare variants is lost.

Genetic architecture

There are forms of genetic architecture within the genome that will play a role in the expression of a phenotype. Some of these, such as epigenetic inheritance [93], can not be modelled or tracked by DNA based markers as they are

chemical alterations of the DNA which cause differential expression to occur. However, others such as: epistasis (discussed further in section 5.2.1.1), pleiotrophy and copy number variation (CNV) can be discovered by using similar models as used in the AS section of this thesis. The latter, CNV, will be the most difficult as modern SNP chips and SNP genotyping technologies can only detect a small proportion of CNVs indirectly (by looking for distortions of signal intensity or inheritance patterns), thus the majority CNVs remain invisible to AS. All of the different forms of genetic architecture mentioned in this section may contribute to the expression of a complex trait, yet to date they have not been analysed sufficiently to highlight the possible variance they may account for.

Linkage Disequilibrium Linkage Association (LDLA) is a two step method used to extract the maximum information from a pedigreed population to detect QTL. Initially an LM approach is conducted to discover broad regions of significance that are inherited throughout a pedigree. These regions are then fine mapped using AS methodology which relies on population LD, enabling the discovery of markers that are closer to a causal variant. This method (LDLA), has been previously shown to be successful in refining confidence intervals (for example: [94-95]) and even discovering casual polymorphisms (for example: [96-97]). Chapter 4 of this thesis follows the same logic. Initially an LM approach is used, which detects broad regions of the genome that are significantly associated the immune traits measured. Following this, further markers are genotyped within these regions (and other regions which were suspected to play a role in the immune response) and an AS conducted in an attempt to fine map the regions of the genome previously shown to be significant.

1.3.3 Quantitative Trait Loci Significance

Due to the nature of both the techniques of QTL detection used in this thesis, multiple testing becomes an issue. In statistical terms, multiple testing becomes a problem when a set of statistical tests (of markers in this case) are calculated simultaneously [69]. For example, if a p-value of 0.05% is used for 1 test (or marker in this example), then the chance of wrongfully rejecting the null hypothesis (type I

error/false positive) is 5%. Thus, if 100 tests are done simultaneously, again with a p-value of 0.05%, then 5 type I errors are expected. To reduce the type I error rate, many multiple testing techniques exist, which all have different attributes [98]. Many are also tailored to be beneficial for certain experimental designs with certain expectations (e.g. normal underlying distributions) [98].

In both the LM and AS methods used in this thesis over 165 markers are tested simultaneously. To correct for this, two methods for multiple testing were used. The initial method was a standard Bonferroni correction [99], which was only used for the AS. The second, permutation testing [100], was used when calculating empirical thresholds during the LM and AS studies.

1.3.3.1 Bonferroni Correction

The Bonferroni correction is a swift multiple correction method designed to obtain an overall significance level [69]. Below is the equation used to calculate the significance threshold when adjusting for multiple testing using the Bonferroni formula:

$$\alpha = 1-(1-\gamma)^{1/n} \quad \approx \quad \gamma/n$$

Where: α is the significance level applied to each of the individual tests (n) and γ is significance level for the entire experiment.

This method of correction is highly conservative when large numbers of tests are used. For example, to achieve an experiment-wide significant marker with a p-value (γ) of 0.05, with the number of tests (n) at 100,000 markers, requires a p-value (α) of 5.00E-07. Thus this has the effect of increasing the frequency of type II errors (false negatives), which can be equally as erroneous as type I errors. As the Bonferroni correction has the expectation that all the markers are not linked. If markers are consistently in LD with each other, then other methods of multiple correction should be used [69].

1.3.3.2 Permutation Testing

Permutation testing is far more robust [69, 100] than the Bonferroni correction method. Using this approach the original analysis is repeated *n* times, each time shuffling the phenotypic and genotypic data and retaining the highest test statistic. As the link between the phenotypes and the genotypes is destroyed, the retained test statistics can be used to calculate 5% and 1% levels significance thresholds.

For example, if 1000 tests were performed and all the test statistics are ordered from lowest to highest, the 10^{th} test statistic would represent the experiment wide (γ) of 1% threshold and the 50^{th} test statistic would represent the experiment wide (γ) of 5% threshold.

This method, however, depending on the complexity of statistical models used, is extremely computationally extensive. For example, *Churchill and Doerge* [100] suggest 10,000 permutations are required to estimate 1% experiment wide significance levels. The time taken to calculate 10,000 permutations in the AS component of this thesis was prohibitively long, thus the Bonferroni correction was chosen for the publication of results.

1.3.4 Marker Assisted Selection

Marker Assisted Selection (MAS) has been used in the cattle industry for at least a decade [101]. MAS is process where a trait of interest is selected, not based upon phenotype alone, but by using a marker that is in linkage with a favourable phenotype [102]. The animals with the favourable allele of the marker are then 'selected' to produce the next generation. This method of selection needs no understanding of the biology and simply uses marker information to select animals based on perceived genetic potential [103]. The SNP markers discovered to be significantly associated to different disease traits, throughout this thesis, could be used, post validation in separate herds measuring the same phenotypes, in large SNP chip arrays in the cattle industry to select animals for better disease resistance.

1.4 Experimental Design

The experimental design undertaken in this thesis, attempted to reduce environmental factors with the aim of maximising detection of genetic affects. Thus the same farm, management team and animal husbandry were used.

1.4.1 The Roslin Bovine Genome (RoBoGen) Herd

The objectives of the RoBoGen project were to map major genes controlling commercially important traits in cattle. The strategy for breeding was to source F1 heifers and bull calves which were progeny of Holsteins mated to one of four pure bred Charolais bulls. From the F1 population, 13 sires were used to create the F2 population and two backcross populations (Charolais backcross (CB1) and Holstein backcross (HB1)) were created (Figure 1.4). Traits were measured on an F2 population of 500 animals; all were originally genotyped for 165 microsatellite markers covering the 29 autosomes of cattle. Successful mapping of QTL, using this herd, to production traits has already been shown [104-106]. In relation to this thesis, the F2 animals were immunised with a peptide derived from Foot and Mouth Disease Virus (FMDV) and vaccinated with a Bovine Respiratory Syncytial Virus (BRSV) vaccine.

1.4.2 The data sets

The FMDV and the BRSV studies were performed on the same animals to minimise the environmental and genetic variance between the two studies. When the animals were vaccinated with the BRSV vaccine IgG1 and IgG2 levels were measured pre and post vaccination. At a later date, the animals were also immunised with a 40-mer FMDV peptide (FMDV15). Once immunised IgG1, IgG2, T cell and Interferon γ (IFN γ) responses were measured post immunisation, over a time course.

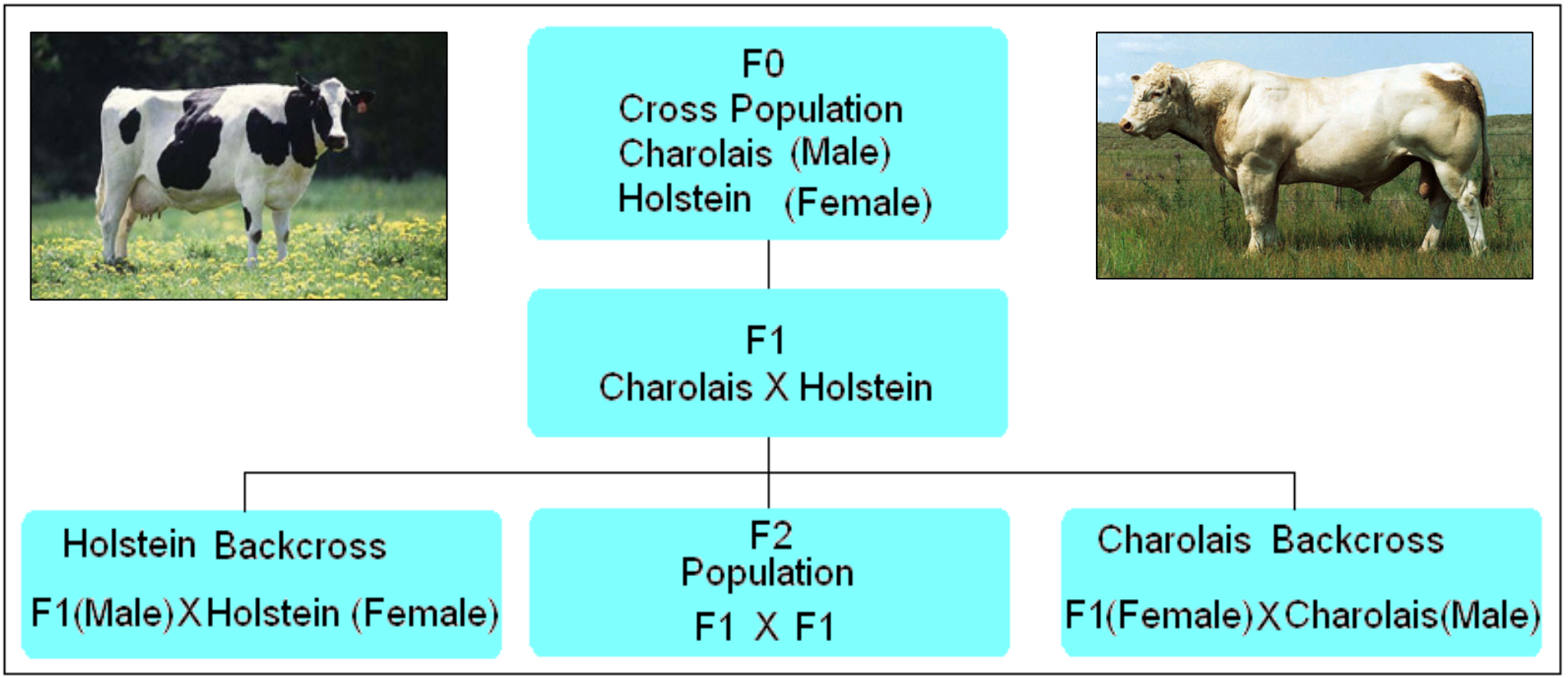


Figure 1.4: The RoBoGen herd and structure.
The F0 founder population consisted of Charolais males and Holstein females. A F2 backcross design was followed.

1.5 Project Overview

The aims of this thesis were to locate and define the genetic component to the observed variability in immune responses pre and post immunisation, identifying markers which are associated with the immune response following immunisation. The markers discovered have the potential to be used in breeding for enhanced disease resistance and may also be used to address poor vaccine efficacy by highlighting potential targets for improving vaccine response, thus aiding vaccinologists to develop next generation vaccines.

The RoBoGen herd was immunised with a FMDV15 peptide and a BRSV vaccine and immune responses were measured over a time course post immunisation. Immune phenotypes and environmental parameters were measured, such as: year of birth (cohort), age at vaccination, weight at vaccination and sex. These parameters enabled detailed statistical analysis of which factors played a significant role post immunisation. The herd was also genotyped to discover the regions of the bovine genome that have a significant effect on the immune response post immunisation.

Aims include:

- Analysing the kinetics of the immune response post immunisation with an FMDV peptide (**chapter 2**)
- Discovery of QTL (via a linkage approach) associated with the immune response post immunisation with an FMDV peptide and BRSV vaccine (**chapters 2 and 3**)
- Discovery of QTL (via a linkage approach) associated with the immune response pre immunisation with the BRSV vaccine. (Chapter 3)
- Comparisons of the immune responses post immunisation to the FMDV peptide and BRSV vaccine (**chapter 3**)
- Genotyping of the RoBoGen herd “under the QTL” previously detected whilst simultaneously using a candidate gene approach (**chapter 4**)
- Further discovery of QTL and genes (via an association approach) associated with the immune response post immunisation with an FMDV peptide and BRSV vaccine (**chapter 4**)

1.5.1 Hypothesis

There is a genetic component to the observed variability in the response to immunisation in cattle. During this thesis I plan to locate and define some of the genetic component, within the RoBoGen population, to the observed variability in response to the immunisations used in this thesis. Furthermore, I will be able to construct a list of regions and genes which are associated with the immune response post immunisation.

Chapter 2

Quantitative trait loci for variation in immune response to a Foot-and-Mouth Disease virus peptide

2.1 Background

As stated in chapter one, variation in the immune response has a genetic component. In this chapter I aim to discover the genetic loci which are associated with an immune response elicited to a Foot and Mouth Disease Virus (FMDV) peptide over a time course. In addition, the kinetics of the immune response will be analysed to detect significant effectors of the immune response.

FMDV is extremely contagious to cloven hoofed animals, posing a particular problem to those on farms due to the close proximity of other cloven hoofed animals. Infection of cattle generally occurs via the respiratory route [107], with the infected animals exhaling the virus in aerosol form [2]. Once the clinical signs which include visible lesions, fever and lameness, are present, viral presence is obvious. However animals are highly infectious before they develop these signs [2], thus in the later stages it is too late to quarantine individual animals. The mortality rate to FMDV is low, generally affecting young animals, where the virus can affect the heart [2]. Due to the effect of the disease it is economically (due to trade restrictions), and on welfare grounds, important to protect animals and farms from outbreaks, such as the 2001 U.K outbreak which cost approximately $13 billion [2].

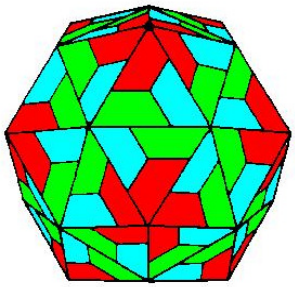


Figure 2.1: A cartoon of the Foot and mouth Disease Virus. Showing the capsid made up of the envelope proteins: VP1 is red, VP2 is green and VP3 is blue. (VP4 is internal).

FMDV is endemic in large parts of the world, though not in the U.K, E.U, U.S.A or Australia. Many countries where FMDV is endemic, routinely vaccinate using inactivated viral vaccines [2]. In contrast, if outbreaks occur in non-endemic countries quarantine and slaughter are the policies used (http://www.oie.int). This however, leaves these countries vulnerable to incursions of exotic pathogens such as FMDV which have occurred in the U.K. Since the 2001 UK outbreak, considerably

more research around the world is directed to developing safer, more efficacious vaccines and other methods of control [108]. In order to achieve this it is clear that greater knowledge of the host response *to*, and interaction *with* FMDV is also required. Considerable animal to animal variation has been reported in the context of FMDV challenge and immunisation with a variety of vaccine constructs including peptides. However the role of host genetics in variation of the response to immunisation has not been generally considered: there has only been one study which suggested that host genetic factors might play a role in response to vaccination with inactivated FMDV vaccines [109].

Foot and mouth disease virus is a member of the picornaviridae family [110]. The virus has a single stranded RNA genome which is non-enveloped. The capsid has icosahedral symmetry with 60 copies of four proteins [2], Vp_1-Vp_4 (Figure 2.1). Currently, there are seven distinct serotypes of the virus: O, A, C, SAT 1-3 and Asia 1 [111] making vaccination difficult as all vaccines against FMDV, to date, are serotype specific. The FMDV peptide (FMDV15) used in this chapter, to elicit an immune response, consists of two sections of the VP1 protein, together encompassing the major neutralising antibody sites [112].

Protection against FMD is generally believed to relate to the levels of neutralising antibody and has been correlated with IgG1 and IgG2 levels [113-114] as well as interferon-γ [115]. In addition, T cell responses play a role in protection against FMDV [116]. The Glass group have shown that there is considerable variation in the immune response to this and related peptides as well as variation in the protection against viral challenge [117-118]. Although previous studies have revealed that polymorphisms in loci within the bovine MHC (BoLA), particularly the class II *DRB3* gene, accounted for some of the variation in immune response [117-119], it seems likely that other genetic factors are also important.

To explore the contribution of the MHC and other loci to variation in immune responses, the work reported in this chapter analyses the variability in the kinetics of FMDV15 specific IgG1, IgG2 and T cell responses during a primary and secondary

response following immunisation with the FMDV15 peptide. The second generation of the RoBoGen herd were also genotyped with 165 microsatellite markers distributed across the bovine genome. The variation in response to the peptide was also correlated with the markers to identify QTL controlling variation in the immune response across a time course.

2.2 Materials and Methods

2.2.1 Acknowledgements

All statistical analysis, within this chapter, was conducted by Richard Leach. The FMDV15 peptide was synthesised by Alistair Douglas, Department of Agriculture for Northern Ireland. All the lab based assays, in this chapter, were completed by Susan Craigmile, the Roslin Institute. Immunisations were done by the Roslin Institute farm staff. John Williams' group at the Roslin Institute completed the genotyping of the RoBoGen herd with the microsatellite markers.

2.2.2 Animals

Immune response measurements were collected from 195 second generation cross heifers (121 from the F2, 43 from the HB1 and 31 from CB1). All female calves were weaned by 36h, segregated from the rest of the herd and raised indoors, initially on milk-replacer then weaned early onto a propriety compound diet. The age of the first immunisation with the FMDV15 peptide ranged from 469-609 days.

2.2.3 Immunisation and sampling

The FMDV15 peptide was chemically synthesized using an ABI 431A peptide synthesiser with FMOC chemistry. Following deprotection and cleavage, it was purified by preparative reverse phase HPLC (Beckman Coulter System Gold HPLC using a Phenomenex Luna C18 column). The peptide consisted of two separate regions (residues 141 to 158 and 200 to 213) of the virus coat protein (VP1) from the O1 Kaufbeuren strain of foot-and-mouth disease virus [112]. The female F2 and backcross heifers were immunised subcutaneously with 1mg FMDV15

peptide/animal emulsified in Freund's incomplete adjuvant at week 0, followed by a boost of 100µg FMDV15 peptide/animal at week 6. Whole blood samples were collected by jugular venipuncture from all of the female F2 and backcross heifers at weeks 0, 1, 2, 4, 8 and 10, post immunisation for IgG analysis (see below) and at weeks 0, 4, 8 and 10 post immunisation for T-cell measurements (see below). For the IgG analysis, the blood samples were allowed to clot, and serum collected and stored at -20 ^{0}C until they were tested for the T cell analysis, blood was collected aseptically into heparin tubes. All experimental protocols were authorised under the UK Animals (Scientific Procedures) Act, 1986.

2.2.4 Phenotypic Data

2.2.4.1 Whole blood T-cell Proliferation assay

The T-cell proliferation assay was carried out essentially as described by Glass et al 1990 [120] with the exception that whole blood was used. Whole blood (150µl) was diluted with 750µl RPMI 1640 supplemented with 25mM HEPES, 2mM glutamine, 10% foetal calf serum, 5 x 10^{-5} M 2-mercaptoethanol either alone as the negative control, 10µg/ml concanavalin A (ConA) (Sigma, UK) as a positive control or 2.0µg/ml FMDV15 peptide (all final concentrations).

Quadruplicate cultures were incubated at 37^{o}C with 5% CO_2 for 6 days. For the last 6 hours the cells were labelled with 0.037MBq ^{3}H-Thymidine per well (GE Healthcare, UK) and uptake assessed by liquid scintillation counting using a 1450 Microbeta (Wallac, now PerkinElmer). The results were expressed as counts per minute of ^{3}H-Thymidine incorporation (mean of quadruplicates).

2.2.4.2 ELISA for detection of FMDV15-specific IgG1and IgG2

FMDV15 peptide specific ELISAs were performed to measure IgG1 and IgG2 isotypes as detailed in Baxter *et al* 2009 [119]. ELISA tests were conducted on the samples from all three cohorts, over a short period following the final sampling, to minimise technical variation. Briefly, Immunolon 2HB plates (Dynex Technologies) were coated with 100µl of 1µg/ml FMDV15 peptide in carbonate/bicarbonate buffer (Sigma), and serum samples were added at predetermined optimal concentrations.

100µl HPR-conjugated sheep anti bovine IgG1 (Cat. No. A10-116P, pre-optimised at 1/20,000 dilution) or IgG2 (Cat. No.A10-116P, pre-optimised at 1/25,000 dilution) (Bethyl Montgomery, Texas, USA) were added as the secondary antibodies and colour developed with Sure Blue Reserve tetramethylbenzidine (TMB) microwell peroxidase substrate 1 component (KPL). Optical density was measured at 450nm on a Victor^2 1420 Multilabel counter (Wallac). Seven serial dilutions of bovine reference serum (Bethyl), containing known concentrations of IgG1 and IgG2 in carbonate/bicarbonate buffer were included on each plate which enabled the IgG1 and IgG2 concentrations to be calculated from simple linear regression (Genstat [121]).

2.2.5 Statistical Analysis

Stimulation Indexes (SI) were calculated for the T-cell proliferation to the FMDV15 peptide and ConA:

$$\text{SI (for the FMDV peptide)} = A_Y / B_x$$

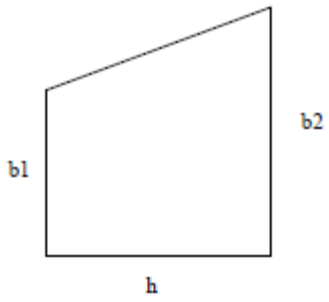
$$\text{SI (for the Con A mitogen)} = A_Z / B_x$$

Where $A_{Y,}$ is the T-cell response to the FMDV peptide, A_Z is the T-cell response to Con A and B_x is the negative control.

The IgG1 and IgG2 concentrations and the T cell SI for ConA and FMDV15 were normalised to obtain a normal distribution and constant variance ($\log_{10}$). Area under the curve (AUC) for each of these four traits was also calculated to provide a single trait that reflected the overall response of the normalised data. For this the trapezoidal rule [122] was used, which is:

$$h(b1+b2)/2$$

Where b1 and b2 are the levels of response and h represents the time elapsed between each measurement:



REML (REsidual Maximum Likelihood) was used, within Genstat [121], to determine which factors were significant within the herd. Sex was omitted from the model as all of the phenotyped animals in this study were female. The final model included sire and dam as random effects; the other effects within the model were, with appropriate degrees of freedom (df), *line* (F2, CB1, HB1; 2d.f) and *cohort* (1, 2, 3; 2d.f). *Age* (age at vaccination; 1d.f) and *calculated weight* (weights of animals at initial vaccination date calculated from regression of animal weights at other time points pre and post vaccination; 1d.f) were both covariates.
Thus the final REML model was:

$$Y_{ijk}= \mu +L+C+\beta_1.m+\beta_2.a+u_j+g_k+e_{ijk}$$

Where: Y_{ijk} is the observed value of the phenotypic trait; μ, population mean; L, the fixed effect of line (3 Lines); C, the fixed effect of cohort (3 Cohorts); β_1, the linear regression on the covariate of age at vaccination m (m = d469-d609); β_2, the linear regression on the covariate of weight a (a= 361-744kg); u_j, the random effect of sire; g_k, the random effect of dam g; e_{ijk}, the residual error e ~ $N(0,I\sigma^2_p)$. The residual variance from the model was used to calculate correlations.

A chi squared test (with 1 df), using the difference of the deviance (-2* log likelihood) of the sire and dam, either in or out of the REML model, was used to calculate the significance of the sire and dam. Wald tests were used to determine the significance of the other effects.

2.2.6 Genetic Markers

Standard phenol-chloroform methods were used to extract DNA from blood samples [123]. A panel of microsatellite markers were genotyped across all individuals in the herd, with a total of 165 microsatellite markers distributed across all 29 autosomes [104]. All of the genotypes were stored in the database ResSpecies [124] and used to build linkage maps with CRIMAP 2.4 [125]. The maps were compared to the latest bovine linkage map [126]. Once compared for consistency, the maps constructed using CRIMAP, were used to conduct the QTL analysis for the immune-related traits (Appendix 2.1).

2.2.7 QTL Analysis

The 24 traits tested in the QTL analysis were: FMDV15 specific IgG1 and IgG2 concentrations at weeks 0, 1, 2, 4, 8, 10 and AUC; and T-cell proliferation to ConA and the FMDV15 peptide at weeks 0, 4, 8, 10 and AUC.

GridQTL [127], internet based software, was used for the QTL analysis. The F2 and backcross module was used which assumes that founder lines are fixed for alternative alleles at QTL loci (although they can be segregating at markers) and implements a least squares analysis. All effects found significant from the previous analysis using REML were included in the model, thus for each time point only significant effects were added to the QTL model. Information content (IC) along the linkage maps was also calculated by the program. Both single and two QTL models with additive and dominance effects were fitted at 1cM intervals along the autosomes (N=29), using the sex averaged genetic map. By setting the 2 founder breeds as: breed 1 = Holstein and breed 2 = Charolais, the positive or negative sign of the additive effects indicated that the Holstein or Charolais allele, respectively, increased the trait values. When the dominance effect had the same sign as the additive effect, this indicated dominance of the Holstein allele over the Charolais allele, whereas if the signs of the additive and dominance effects were opposite, the Charolais allele was dominant.

In total QTL were sought for 24 traits using the 165 microsatellite markers spread across all 29 autosomes of the bovine genome. Significance thresholds were calculated by permutation analysis with 1000 permutations [100]. Four significance levels were used: chromosome-wide 5% and 1% and genome-wide 5% and 1%.

2.2.8 Refining QTL

The QTL detected at the 5% chromosome-wide significance level and above were included in the model and the genome rescanned for further QTL. By adding the initial QTL as background effects, the variance caused by them is removed, thus potentially revealing previously undetected QTL. In cases where more than one QTL was found for the same trait on the same chromosome, a 2 QTL model was performed by fitting two QTL simultaneously and re-analysing the data. A forward and backward selection interval mapping approach was used to check whether QTL moved, relative to other QTL of the same trait [128], however none did. These refining methods were repeated, until no further QTL were detected. Finally, bootstrap analysis was performed, using 1000 repeat samples, for all chromosomes where a significant QTL was detected at the 5% chromosome-wide threshold [83] to estimate the 95% confidence intervals for location of the QTL, except for the 2 QTL models which were not bootstrapped.

2.3 Results

The 195 female F2, Holstein back-cross and Charolais back-cross animals of the "RoBoGen" population were immunised with the FMDV15 peptide and the resulting antibody and T cell responses were measured across time. The T cell responses to a T cell mitogen, Concanavalin A (ConA) were also measured across time. The whole herd (males and females in the F0 to F2 generation, in total 984 animals) was genotyped with 165 microsatellite markers.

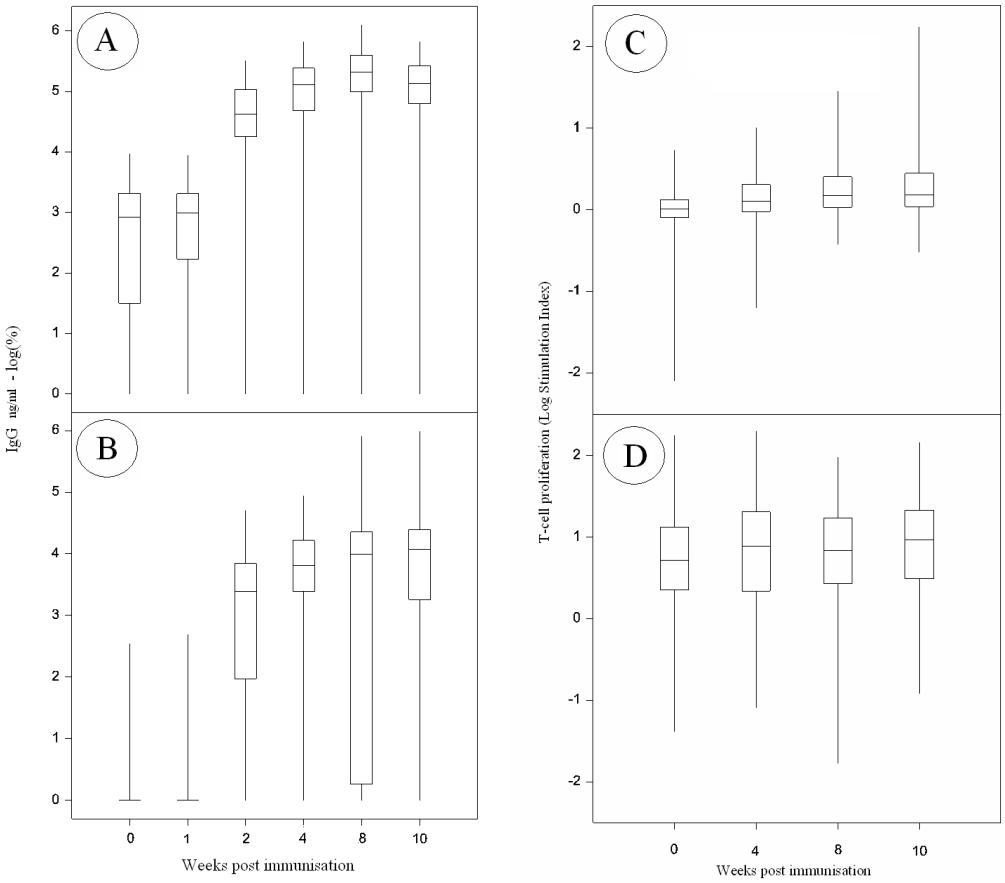


Figure 2.2: Variation in IgG isotype and Tcell responses
Box plots depicting IgG and T cell responses. In all diagrams: Median (central horizontal line), quartiles (outer horizontal lines) and range (outer vertical lines) shown. All data is log10 transformed.
On the left: FMDV15 peptide specific IgG1 (A) and IgG2 (B) levels following immunisation at weeks 0 and 6 of 195 female cattle.
On the right: T cell proliferation (stimulation index) across time following immunisation with FMDV peptide. 2ug/ml FMDV15 peptide (C),and 10ug/ml Con A (D).

2.3.1 Kinetics of responses to immunisation

A humoral and cell-mediated response to the FMDV15 peptide was detected in the majority of animals (Figure 2.2). From week 2, responses were significantly different from week 0 for anti FMDV15 peptide specific IgG1 and IgG2, and from week 4 for the T cell proliferative response to the FMDV15 peptide. Following a second immunisation, most animals exhibited an increase in their response. The mean values for the IgG1 responses (Standard Deviation, SD) for weeks 0, 1, 2, 4, 8, 10 and AUC (area under the curve, to provide a single trait that reflected the overall response, see methods.) were 1.2 (1.4), 1.3 (1.4), 69.7 (66.1), 167 (144.4), 291.7 (269.2), 176.1 (141.6) and 1658.4 (1381.3) μg/ml respectively. The IgG2 responses for weeks 0, 1,

2, 4, 8, 10 and AUC were 0.01 (0.04), 0.01 (0.05), 5.6 (8.8), 13.1 (17.3), 24.3 (64.7), 23.5 (71.7) and 144.4 (302.3) μg/ml respectively. The means for the T cell stimulation index of response to the FMDV peptide at weeks 0, 4, 8, 10 and AUC were 1.131 (0.58), 1.8 (1.5), 2.6 (3.7), 4.6 (14.6), 21.9 (27.3) SI.

Correlations between the time points and between traits (Table 2.1) revealed that there was a high correlation between the antibody levels at week 0 and 1 ($r = 0.88$, $p < 0.01$ for IgG1; $r = 0.61$, $p < 0.01$ for IgG2). Most of the significant correlations were positive. The early IgG1 response (week 2) correlated with later responses at week 4 ($r = 0.31$, $p < 0.01$) and 8 ($r = 0.26$, $p < 0.01$) and the pre-boost response at week 4 correlated with the post-boost response at week 10 ($r = 0.69$, $p < 0.01$). In addition responses at weeks 8 and 10 were also highly correlated ($r = 0.38$, $p < 0.01$). The IgG2 response showed similar correlations in so far that earlier responses correlated with later responses, with the strongest correlations ($r > 0.4$, $p < 0.01$) seen between weeks 2 and 4, weeks 4 and 8, and weeks 8 and 10. Similarly, the pre-boost T cell response to the FMDV15 peptide at week 4 correlated with the post-boost response at weeks 8 and 10 ($r = 0.224$ and $r = 0.155$; $p < 0.01$), with the highest correlation between weeks 8 and 10 ($r = 0.57$, $p < 0.01$). The ConA T cell responses correlated moderately across all time points with $r > 0.2$ ($p < 0.01$), except between weeks 0 and 4 which had an $r = 0.12$, which was not significant ($p = 0.1$).

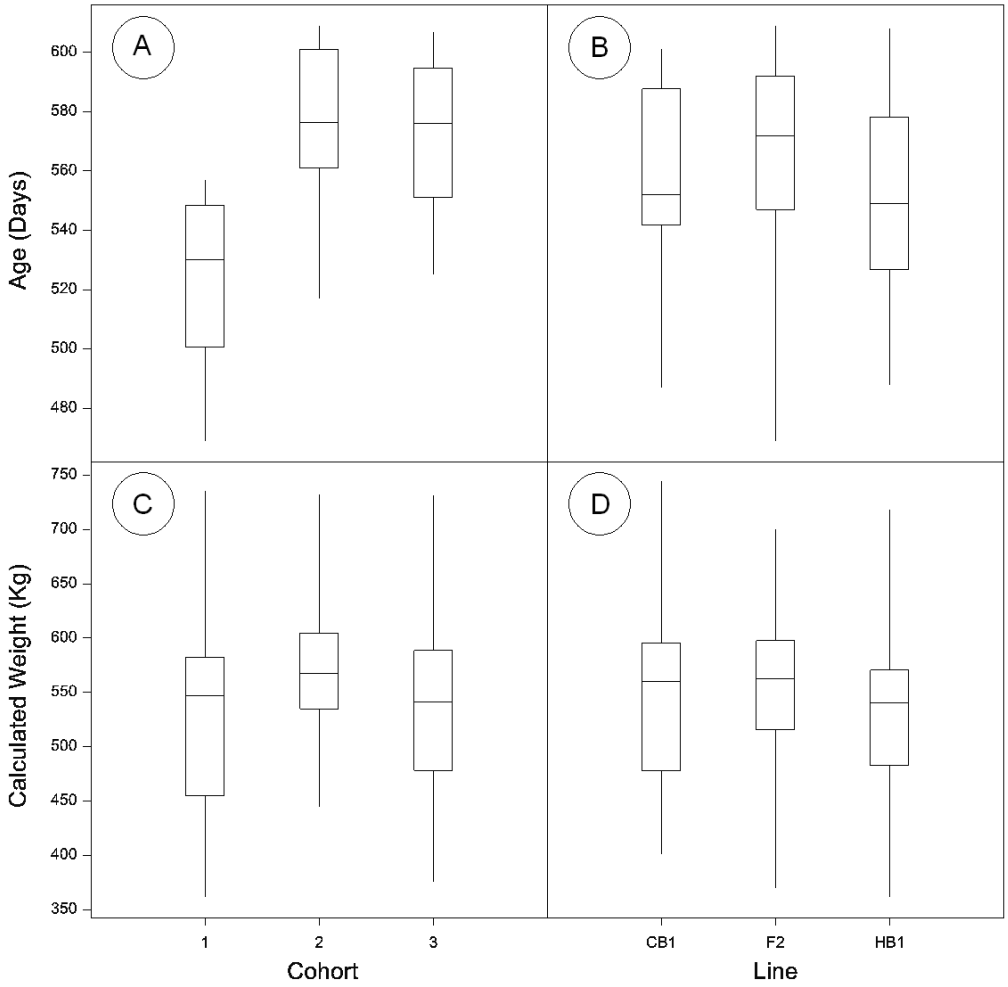


Figure 2.3: Box plots depicting the distributions of the fixed effects and covariates
In all diagrams: Median (central horizontal line), quartiles (outer horizontal lines) and range (outer vertical lines) shown.
The distribution of age (A and B) throughout the cohorts and lines, respectively.
The distribution of calculated weight (C and D) throughout the different cohorts and lines, respectively.

Furthermore, correlations were found between some of the different trait types (Table 2.1). The IgG1 and IgG2 responses were moderately correlated at the same weeks from 2 to 10 (r at least 0.21, all values $p<0.01$), with the exception of the correlation of IgG1 week 4 and IgG2 week 2. No significant correlations were seen between the anti FMDV15 T cell response and IgG1 responses. However, some weakly significant correlations were seen between the T cell response and the IgG2

response. In addition the T cell response to the FMDV peptide correlated with the ConA response, with the strongest correlations seen at the same weeks 4, 8 and 10 (r >0.43, p<0.01).

	IgG1_week_0	IgG1_week_1	IgG1_week_2	IgG1_week_4	IgG1_week_8	IgG1_week_10	IgG2_week_0	IgG2_week_1	IgG2_week_2	IgG2_week_4	IgG2_week_8	IgG2_week_10	SI_FMDV_week_0	SI_FMDV_week_4	SI_FMDV_week_8	SI_FMDV_week_10	SI_CA_week_0	SI_CA_week_4	SI_CA_week_8	SI_CA_week_10
IgG1_week_0																				
IgG1_week_1	0.88																			
IgG1_week_2	0.07	-0.02																		
IgG1_week_4	0.16	-0.09	0.31																	
IgG1_week_8	0.04	-0.03	0.26	0.09																
IgG1_week_10	-0.11	0.09	-0.04	0.69	0.37															
IgG2_week_0	-0.05	0.04	0.02	0.07	0.00	-0.02														
IgG2_week_1	0.12	-0.09	-0.01	-0.02	-0.02	0.00	0.61													
IgG2_week_2	-0.10	0.02	0.31	-0.01	-0.02	-0.07	0.02	0.07												
IgG2_week_4	0.07	-0.03	-0.07	0.14	-0.15	-0.01	-0.07	0.10	0.41											
IgG2_week_8	-0.03	0.00	-0.03	-0.06	0.21	-0.05	0.01	-0.05	0.16	0.43										
IgG2_week_10	0.02	0.01	0.03	-0.18	-0.02	0.24	0.02	0.02	-0.02	0.17	0.46									
SI_FMDV_week_0	-0.03	0.03	-0.05	-0.13	-0.04	0.09	0.03	-0.07	-0.07	0.02	0.15	-0.11								
SI_FMDV_week_4	0.08	-0.04	-0.06	0.05	-0.05	-0.06	0.05	-0.19	0.01	0.03	0.04	0.10	-0.02							
SI_FMDV_week_8	-0.06	0.08	0.10	-0.05	0.01	0.02	0.01	0.12	-0.12	-0.06	0.14	-0.01	0.05	0.22						
SI_FMDV_week_10	0.11	-0.14	-0.14	-0.09	0.11	0.08	0.07	-0.10	0.18	0.00	-0.06	0.02	0.15	0.16	0.57					
SI_CA_week_0	-0.04	0.02	-0.06	0.10	0.12	-0.02	-0.15	0.26	0.04	0.02	-0.09	-0.06	0.32	0.12	-0.08	-0.15				
SI_CA_week_4	-0.05	-0.01	0.00	-0.10	0.04	0.01	-0.05	0.20	0.00	0.05	0.03	-0.05	-0.04	0.43	-0.13	0.04	0.12			
SI_CA_week_8	0.07	-0.10	-0.03	0.02	-0.03	-0.01	0.02	-0.08	0.05	0.05	-0.14	0.15	-0.02	-0.11	0.49	-0.41	0.21	0.21		
SI_CA_week_10	-0.12	0.12	0.08	0.04	-0.10	0.00	0.07	-0.10	-0.07	-0.06	0.11	-0.08	-0.16	-0.17	-0.27	0.47	0.23	0.35	0.54	

Table 2.1: Correlation matrix of traits
Residuals for the REML model were used to calculate the correlations between each week and trait. r values that are +/- 0.2 are coloured grey and are significant to p<0.05

2.3.2 Analysis of fixed and random effects

As reported above considerable variation was observed between animals for all three immune parameters, IgG1, IgG2 levels and T cell proliferation (Figure 2.2). The greatest variation among animals for both isotypes was seen at week 8 at which time the IgG2 response, was significantly greater than that of IgG1 (p<0.001). In addition, the proliferative response to ConA varied among animals and across time points following immunisation (Figure 2.2). The T cell proliferative response to the FMDV15 peptide (Figure 2.2) showed less variation among animals across the time points than the T cell proliferation to Con A. The skew (Figure 2.2) in the T cell response to the FMDV15 peptide changed over time, from a negative skew to a positive skew as more animals made a response in the later time points.

When week was included as an interaction within the REML model, cohort was significant for both the T cell response to the FMDV peptide (p<0.001) and the T cell response to Con A (p<0.001). Further, weight at vaccination was found to be

significant for both T cell response to the FMDV peptide (p=0.015) and the T cell response to Con A (p=0.019), with immune levels increasing with weight. Age at initial vaccination was only significant for the IgG2 responses to the FMDV peptide (p=0.005) and IgG2 levels increased with age.

When the traits were analysed week by week (Table 2.2) cohort was significant throughout for each trait, with the exception of the IgG2 response to the FMDV15 peptide. The effects of age and weight also differed: age at vaccination was significant at weeks 0 and 1 for IgG1 responses and only at week 1 for IgG2 responses. No significant age effect was found for either of the T cell proliferation responses. Weight was found to be significant for T cell proliferation to the FMDV peptide at week 8 post vaccination (p=0.049). Line (F2, CB1, HB1) was not a significant factor for any traits. The distributions of age and weight within the cohorts and lines are shown in Figure 2.3. No significant interactions were discovered, with the exception of an age difference in cohort 1. This however, was only significant at week 0 and week 1 post vaccination for the IgG1 and IgG2 responses.

Sire and dam effects varied throughout the study (Table 2.2). No significant sire effects were seen for IgG1 responses and only some dam effects were detected, however most dams only had one calf. The IgG2 response had no significant dam effects whereas sire was significant from weeks 2 to 10 inclusive (all $p<0.05$, with weeks 8 and 10 being $p<0.005$). The T cell proliferation responses showed no significant association with sire or dam, with the one exception of a dam effect at week 0 for the Con A response (p=0.005).

Trait	Week	Sire	Dam	Cohort	Age	Weight
	0			0.001	0.001	
	1		0.05	0.001	0.001	
	2			0.001		
IgG1	4		0.05	0.001		
	8		0.05	0.032		
	10			0.001		
	AUC		0.001	0.001		
	0					
	1			0.001	0.012	
	2	0.01				
IgG2	4	0.05				
	8	0.005		0.031		
	10	0.005				0.025
	AUC	0.005				
	0		0.005	0.001		
	4			0.001		
Con A SI	8			0.001		
	10			0.001		
	AUC			0.001		0.02
	0			0.018		
	4			0.001		
FMDV SI	8			0.003		0.049
	10			0.001		
	AUC			0.001		0.012

Table 2.2: Factors used in the REML model and their significance
Only p-values <0.05 are shown for each week for all four traits. Line was not significant for any trait at any time point.

2.3.3 Overall QTL Results

A total of 77 QTL were identified for all 4 of the trait types (IgG1 and IgG2 concentrations; T cell proliferation to Con A and FMDV15 peptide) studied (Table 2.3). Of these, 11 were above the 1% chromosome significance threshold (but below the genome wide 5% significance threshold), 2 were above the genome wide 5% significance threshold (but below the genome wide 1% significance threshold) and 1 was above the 1% genome wide significance threshold. The initial analysis revealed 54 QTL, and a further 23 were located by fitting each QTL as a background effect. The majority of the confidence intervals (CI) (Appendix 2.2) were large with an average of 72.8cM., calculated across all QTL above the 5% chromosome wide significance level. The CI reflected the estimated QTL effect size, the number of animals used in the study and the density of the markers used. The average

phenotypic variance (corrected for fixed effects) accounted for in this study across all QTL above the 5% chromosome wide threshold was 5.8%. A total of 46 significant additive effects were found (Table 2.3). In 18 of these instances the Charolais allele increased the traits' values, whereas the remaining 28, the Holstein allele increased the traits' values. Dominance effects were significant in 42 QTL.

Each of the four trait types (IgG1 response; IgG 2 response; T cell response to FMDV and T cell response to Con A), were clearly under polygenic control (Table 2.3 and Table 2.4), and no QTL were found that controlled all traits. Of the 77 QTL detected, the majority (51) played a role in determining the FMDV15 specific antibody responses, whereas fewer (26) controlled the T cell response to FMDV15 and Con A.

With few exceptions, different QTL were detected at different time points. The majority of QTL appeared to be specific for a given trait type or for early or late time points, suggesting that T cell and antibody response must be, in the main, under the control of different genes (Table 2.4). A few chromosomal regions appeared to control more than one type of response in particular QTL on BTA 20 (Figure 2.4 and Table 2.3), BTA 23 (Figure 2.4) and BTA 25 (all 3 BTA shown in Table 2.3) impacted on the greatest number of traits (9, 9 and 7 respectively). QTL on BTA 4, 19, 20 and 23 all played a role in both the primary and secondary responses (Table 2.4), whereas QTL on BTA 20, 23, 24 and 25 all affected the FMDV15 specific IgG1 and IgG2 at several time points (Table 2.4), although the QTL located on BTA 25 specifically influenced the primary IgG1 and IgG2 responses and not the secondary antibody responses. In contrast the QTL on BTA 24 was detected for the secondary responses of both antibody isotypes but not the primary responses. Interestingly, the QTL on BTA 23 influenced earlier time points for the IgG1 response, and not the IgG2 response (Table 2.4 and Figure 2.5). A QTL with a strong additive effect ($p<0.01$) was identified on BTA 23 associated with the IgG2 response at week 10, where the additive effect of the Holstein allele was an increase of 10.5 μg/ml. IgG2 responses in week 8 on BTA 12 and 15, both showed large dominance effects of 17.4 μg/ml and 11.0 μg/ml ($p<0.01$ and $p<0.1$, respectively).

Very little overlap was observed in the chromosomal regions controlling the T cell response to the FMDV 15 immunising antigen, and the T cell response to the T cell mitogen, ConA, with the exception of QTL on BTA 6 and BTA 29. In addition, very little overlap was seen between QTL controlling the T cell responses and the antibody responses, with the exception of the QTL on BTA 20, which appeared to play a role at several time points for both antibody isotypes as well as the T cell response at the initial immunisation time point. Indeed the QTL with the highest F value (10.07; 1% genome wide significance) in the study was for the AUC measurement for the anti-FMDV15 IgG1 response, and was located on BTA 20, where the clusters of QTL controlling IgG1 and IgG2 traits are also located. This QTL alone accounted for 9.8% of the variance for the overall IgG1 response. Other highly significant QTL included a QTL on BTA 24 for the IgG1 response at week 8 and T cell response to Con A at initial immunisation on BTA 6 (both 5% genome wide significance).

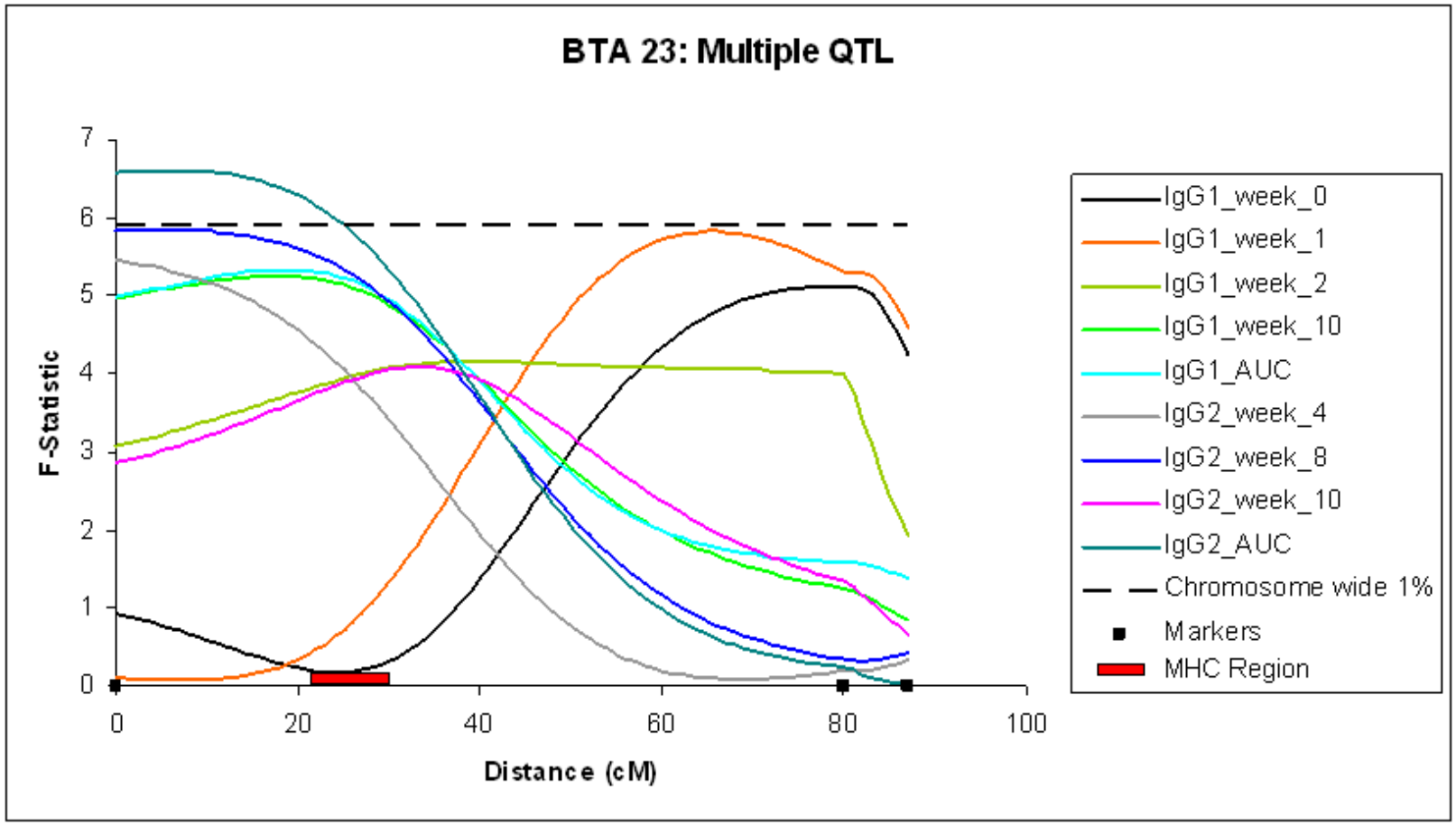


Figure 2.4: BTA 23: Multiple QTL
F-statistic profile and support intervals for IgG 1 and IgG 2 responses (AUC = area under curve) elicited by FMDV15 peptide, located on chromosome 23. The dashed horizontal line represents the threshold of the 1% chromosome wide significance (F =5.90). The red rectangle on the x-axis shows the approximate location of the MHC region.

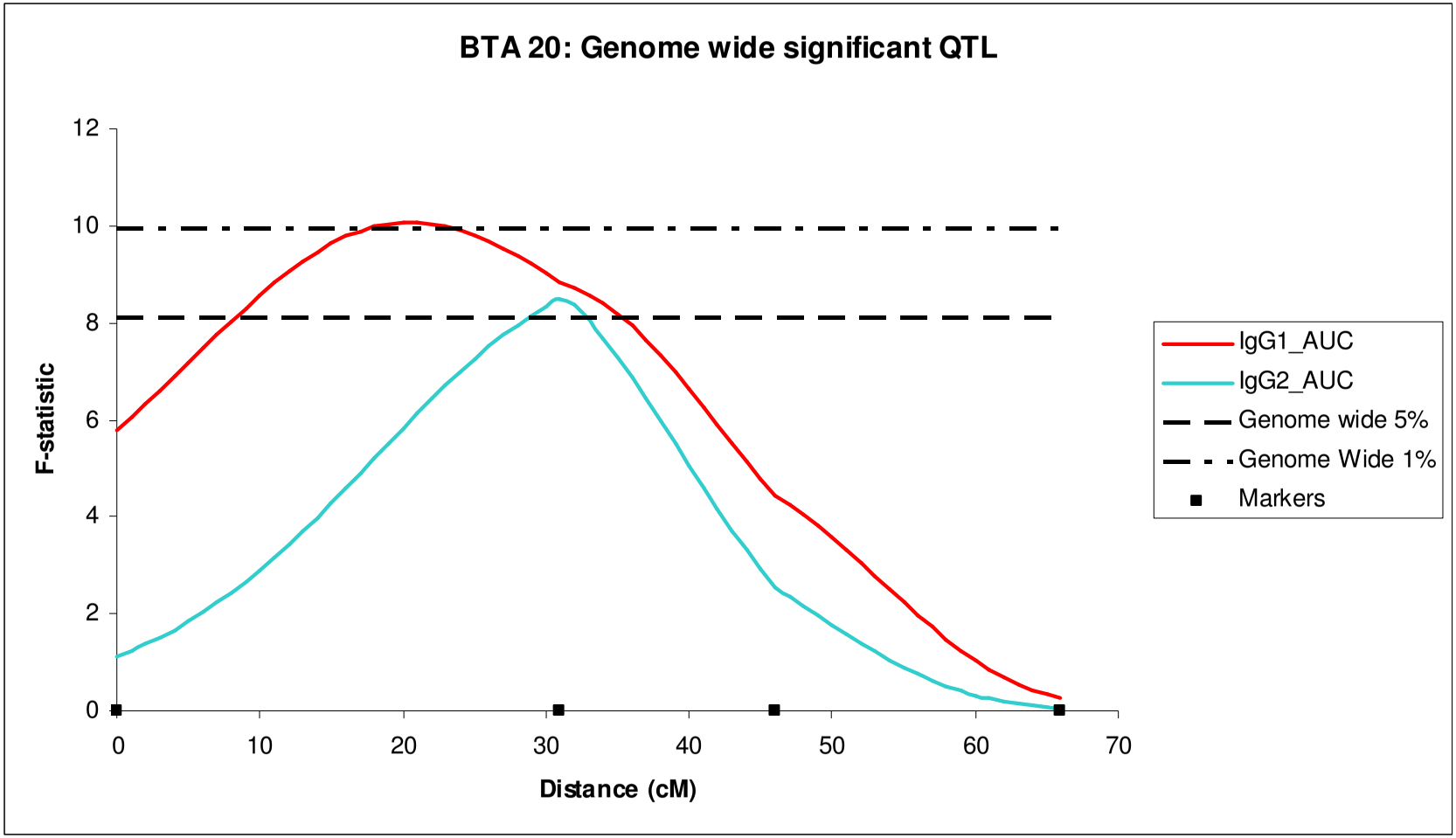


Figure 2.5: BTA 20: Genome wide significant QTL
F-statistic profile for IgG1 and IgG2 responses (AUC = area under curve) elicited by FMDV15 peptide, located on chromosome 20. The dot-dashed horizontal line represents the threshold of the 1% genome wide significance (F = 9.94). The dashed line represents the threshold of the 5% genome wide significance (F = 8.10).

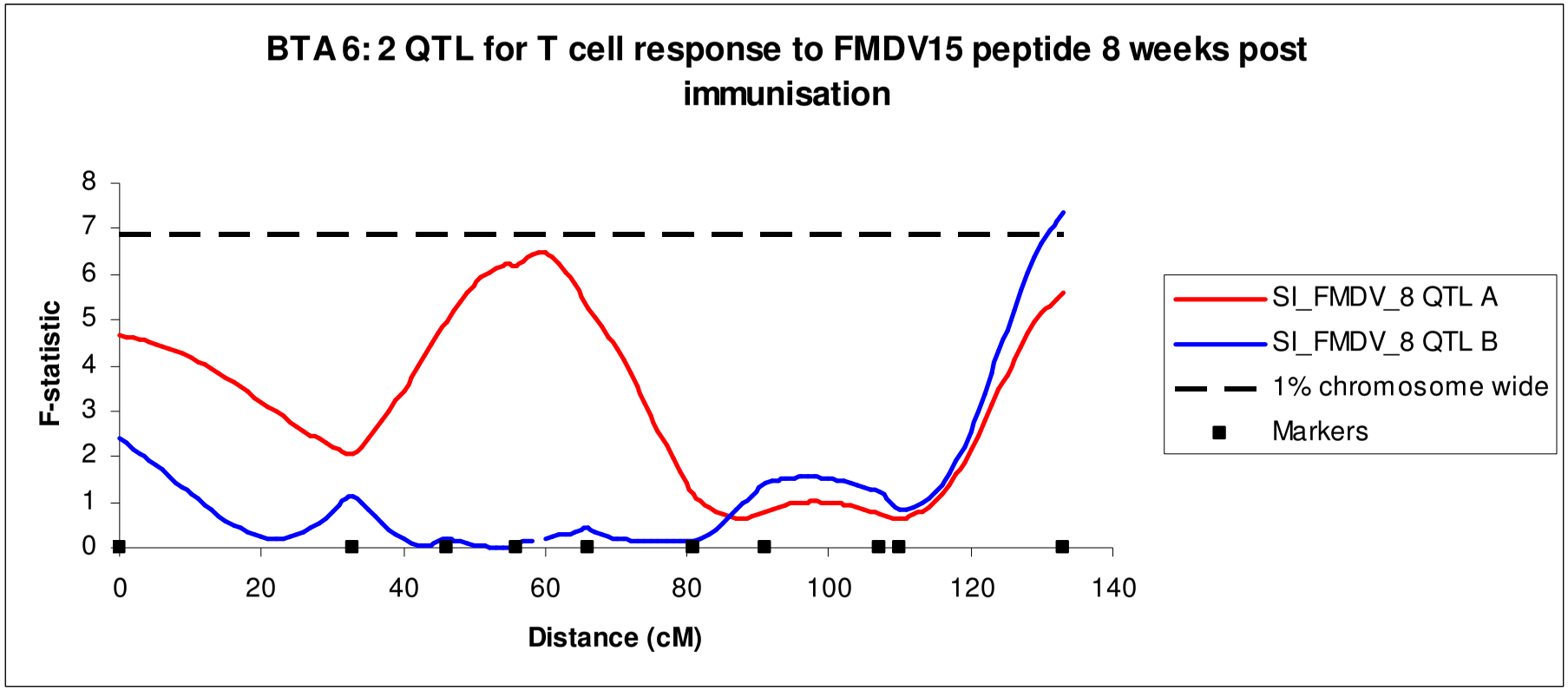


Figure 2.6: BTA 6: 2 QTL for T cell response to FMDV15 peptide 8 weeks post immunisation
F-statistic profiles for two QTL on BTA 6 for the T cell proliferation to FMDV15 peptide at week 8. The peak of QTL A occurs at 59 cM. Following the addition of QTL A as a background effect, QTL B was revealed. The peak of QTL B is at 133cM. The dashed horizontal line is the 1% chromosome wide significance level (F=6.85).

2.3.4 2 QTL Model

Following the initial analysis, each QTL was added as background effect and 2 QTL models were run. This analysis identified 3 significant 2 QTL models (Table 2.3). These included the T cell response to Con A at Week 0 on BTA 5; T cell responses to the FMDV15 peptide at Week 8 (Figure 2.6) and AUC, both on BTA 6. Each of these pairs of QTL on single chromosomes had at least two markers separating them.

Chromosome [1]	Trait [2]	cM [3]	F [4]	a [5]	d [5]	Var% [6]
2	IgG1_week_10	38cM	5.60	-0.21**	0.26**	5.71
3	IgG2_week_2	58cM	5.93	0.66**	0.40	6.03
4	IgG1_week_1	31cM	5.35	-0.63**	0.34	5.46
4	IgG1_week_8	20cM	5.17	0.12	0.17**	1.18
4	IgG1_week_10	27cM	4.92	0.23	-0.47*	5.05
4	IgG1_AUC	28cM	6.37*	0.11	-0.43*	6.64
4	SI_FMDV_Week_0	67cM	4.79	0.00	-0.14**	4.92
5	SI_CA_Week_0	68cM	8.10*	-0.13*	0.24**	8.05
5	SI_CA_Week_0	100cM	5.76	0.22**	-0.14	6.11
5	SI_CA_Week_10	76cM	5.26	-0.05	0.23**	5.38
5	SI_CA_10ug_AUC	69cM	5.89	-0.06	0.17**	6.04
6	SI_CA_Week_0	30cM	8.69**	0.26***	0.08	8.59
6	SI_FMDV_Week_8	59cM	6.49	0.14**	-0.08	6.56
6	SI_FMDV_Week_8	133cM	7.35*	0.04	-0.20***	7.51
6	SI_FMDV_Week_10	3cM	6.61	0.12**	-0.15*	6.67
6	SI_FMDV_AUC	2cM	7.91*	0.10***	-0.07	7.96
6	SI_FMDV_AUC	133cM	6.21	0.03	-0.13**	6.56
7	IgG2_week_8	0cM	6.24	-0.85***	0.13	6.58
7	SI_CA_Week_10	0cM	4.89	0.05	0.28**	5.02
7	SI_FMDV_Week_4	54cM	5.45	-0.11**	-0.09	5.62
9	IgG1_week_0	40cM	5.29	-0.2948*	0.35	5.41
9	SI_FMDV_Week_10	63cM	4.92	0.05	-0.23**	5.05
11	IgG1_week_0	0cM	5.26	0.25	-0.61*	5.38
12	IgG2_week_8	17cM	5.44	-0.03	1.24***	5.56
13	IgG1_week_1	0cM	5.08	0.04	-0.54**	5.20
14	IgG2_week_0	2cM	4.85	0.12	-0.28**	4.98
15	IgG2_week_8	13cM	5.16	-0.08	1.03**	5.28
15	IgG2_AUC	27cM	5.39	-0.08	0.67**	5.51
16	IgG2_week_2	76cM	6.51	-0.71***	-0.16	6.58
16	SI_FMDV_AUC	64cM	4.42	-0.06*	-0.07*	4.61
18	SI_CA_Week_10	22cM	7.02*	-0.20***	0.10	7.06
18	IgG2_week_1	57cM	5.23	-0.10	0.34**	5.40
19	IgG2_week_2	28cM	4.93	-0.50*	-0.62*	5.06
19	IgG2_week_4	34cM	5.64	-0.73***	-0.32	5.75
19	IgG2_week_8	50cM	5.28	-0.81**	-0.25	5.63
19	SI_CA_Week_8	32cM	8.57*	0.25***	-0.07	8.48
19	SI_CA_Week_10	32cM	4.54	0.15*	-0.13	4.67
19	SI_CA_10ug_AUC	28cM	4.42	0.13**	-0.01	4.61

20	IgG1_week_2	28cM	4.74	-0.03	0.48**	4.87
20	IgG1_week_4	36cM	7.86*	0.20**	0.35**	7.83
20	IgG1_week_8	9cM	6.49*	0.19	0.64**	6.56
20	IgG1_week_10	23cM	5.37	0.09	0.38**	5.48
20	IgG1_AUC	20cM	10.07**	0.12	0.39***	9.82
20	IgG2_week_4	32cM	7.48	0.52**	0.65*	7.48
20	IgG2_week_8	25cM	8.46	0.76**	0.87*	8.38
20	IgG2_week_10	31cM	6.23	0.33	0.82**	6.31
20	IgG2_AUC	31cM	8.5*	0.40**	0.67**	8.41
20	SI_FMDV_Week_0	61cM	7.95*	0.05	-0.18***	7.92
21	IgG1_week_2	69cM	4.96	-0.22*	0.38*	5.09
21	SI_CA_10ug_AUC	84cM	4.10	-0.14**	-0.01	4.28
23	IgG1_week_0	78cM	5.11	-0.19	-0.54**	5.24
23	IgG1_week_1	65cM	5.83	-0.13	-0.87***	5.92
23	IgG1_week_2	39cM	4.16	0.37	-0.53	4.31
23	IgG1_week_10	18cM	5.25	0.37**	0.03	5.37
23	IgG1_AUC	18cM	5.34	0.29**	0.06	5.46
23	IgG2_week_4	0cM	5.45	0.63**	-0.18	5.57
23	IgG2_week_8	5cM	5.85	0.88***	-0.18	5.94
23	IgG2_week_10	33cM	4.08	1.02**	0.77	4.23
23	IgG2_AUC	6cM	6.61*	0.66***	-0.18	6.67
24	IgG1_week_8	35cM	7.83**	0.58***	0.32	7.80
24	IgG1_week_10	33cM	4.18	0.27**	0.06	4.32
24	IgG1_AUC	33cM	6.23	0.25***	0.12	6.31
24	IgG2_week_10	21cM	5.09	0.69**	0.61	5.32
24	SI_CA_Week_4	34cM	5.46	-0.05	0.28**	5.63
25	IgG1_week_0	0cM	4.48	0.34*	0.28	4.62
25	IgG1_week_1	0cM	4.88	0.29*	0.35*	5.01
25	IgG1_week_2	33cM	4.42	0.05	-0.35**	4.66
25	IgG2_week_1	15cM	4.48	0.18**	-0.11	4.62
25	IgG2_week_2	34cM	4.65	-0.14	-0.73**	4.78
25	IgG2_week_4	34cM	4.85	-0.14	-0.70**	4.98
25	IgG2_AUC	34cM	5.28	-0.04	-0.62**	5.40
25	SI_CA_Week_10	17cM	5.79	-0.11*	0.19**	5.89
26	IgG1_week_1	21cM	4.90	-0.30*	0.35	5.25
27	IgG2_week_4	7cM	4.61	-0.57**	-0.33	4.75
29	SI_CA_Week_0	0cM	5.34	0.15**	-0.11	5.69
29	SI_FMDV_Week_8	7cM	4.90	-0.10*	0.12	5.03
29	SI_FMDV_Week_10	0cM	4.38	-0.11**	0.08	4.61

Table 2.3: All QTL located in this study

1. Chromosome: the chromosome number of the QTL. Underlined if 2 QTL model.
2. Trait: each trait is shown as follows: trait type (IgG1; IgG2; SI_FMDV = T cell proliferation to the FMDV peptide; SI_CA = T cell proliferation to Concanavalin A), followed by week post immunisation.
3. cM: the position the QTL is on the chromosome, in centiMorgans.
4. F: the F-statistic for each QTL. Significance level: all are at least 5% chromosome wide, * =p<1% chromosome wide, **=p<5% genome wide and ***=p<1% genome wide.
5. "a" and "d" are the additive and dominance effect, respectively, of each QTL, * =p<5%, **=p<1% and ***=p<0.01%.
6. Var%: the phenotypic variance explained by the QTL.

Clusters of related traits have been boxed, 6 in total.

2.3.5 Clusters

Although a few chromosomes (BTA 2, 3, 11, 12, 13, 14, 26 and 27) only appeared to have QTL affecting an individual immune-related trait at one time point, several chromosomes had QTL affecting a trait across time. All chromosomes with greater than 3 QTL for the same trait across time (e.g. IgG1 weeks: 0, 2 and 4) with overlapping CI are shown with outline boxes in Table 2.3, and are referred to as "clusters". There were 6 such clusters located on BTA 4, 5, 6, 20, 23 and 25 (Figure 2.4 and Figure 2.5 also Table 2.4). The most significant QTL in the study was located within the clusters of QTL for IgG1 and IgG2 responses on BTA 20 (Figure 2.5). This region also influenced the T cell response to FMDV15 at the time of immunisation. BTA 23 contained two QTL clusters for IgG1 and IgG2 responses. All of the clusters, with the exceptions of the clusters on BTA 4 and BTA 5, had additive effects showing Holstein alleles increased the phenotype.

		BTA number [3]											
Trait [1]	Week [2]	9	11	23	25	13	26	4	21	20	24	2	
IgG1	0	x4	x	x	x								
	1			x	x	x	x	x					
	2			x	x				x	x			
	4									x			
	8							x		x	x		
	10			x				x		x	x	x	
		14	18	25	3	16	19	27	20	23	7	12	15	24
IgG2	0	x												
	1		x	x										
	2			x	x	x	x							
	4			x			x	x	x	x				
	8						x		x	x	x	x	x	
	10								x	x				x
		4	20	6	7	29	9							
T-cell FMDV	0	x	x	x										
	4				x									
	8			xx		x								
	10			x		x	x							
		29	6	5	24	19	25	7	18					
T-cell ConA	0	x	x	xx										
	4				x									
	8					x								
	10			x		x	x	x	x					

Table 2.4: Chromosomal positions of all QTL
1. Trait = immune response type to the FMDV15 peptide
2. Week post immunisation
3. Bos Taurus Autosome (BTA) number
4. Each 'X' represents the presence of a QTL, 'XX' represents 2 QTL. All are at least 5% chromosome wide significant
(AUC results are omitted as they are representative of the overall response across time.)

2.4 Discussion

This study has revealed that both the humoral and cell mediated immune response to a relatively simple 40-mer peptide are under the control of a considerable number of chromosomal loci. This study is also unique in that it was possible to measure both a primary and secondary immune response across time and thus detect QTL that influenced different phases of the immune response (Table 2.4). Most other studies in livestock mammals which have investigated immune or infectious disease related QTL have typically focused on infectious disease related phenotypes and generally identified QTL from animals exposed to natural infections and not experimental challenge. This is particularly true for cattle where QTL have been reported for disease resistance in general [129] or resistance to a pathogen [5, 55, 130-132]. In all of these studies the phenotypes relied on veterinary observations. In contrast, the phenotypes described in the present study were all obtained using a standardised experimental protocol and a uniform dose of a specific antigen. This may, at least in part, explain the large number of QTL detected. The outcomes of disease resistance or vaccine induced protection are likely to depend on a range of immune-related mechanisms. The present study concentrated on some of these underlying traits, namely antibody and cell mediated responses.

Unexpectedly, a number of QTL were detected for the 0 time point for both antibody and T cell proliferation. It is difficult to interpret these results as the animals were naïve to the FMDV peptide. In a previous study it was noted that the control values for T cell proliferation were under genetic control [133], and thus the QTL for the T cell proliferation to the FMDV peptide at time 0, may be related to the inherent ability of T cells in culture to proliferate. Measurements of cellular immunity are inherently more difficult to conduct than measurements of humoral immunity, partly because cell based assays have to be conducted with fresh cells. For the work reported here a high-throughput system had to be devised to measure the cellular immune response to the FMDV peptide in large numbers of animals. Whole blood rather than purified peripheral blood mononuclear cells was used, and T-cell proliferation was measured on day six for logistical reasons, which may have resulted in greater variation compared to the proliferation response by purified

peripheral blood mononuclear cells. A further factor that may explain the high 0 time values is that the FMDV peptide contains an "RGD" motif which enables the FMDV virus to bind to integrins on the cell surface to facilitate virus entry [134]. Fluorescently labelled FMDV peptide has been shown to bind to a wide variety of cells (Glass unpub.obs.) potentially through integrin binding. In addition it is possible that there are soluble molecules in serum that bind to the "RGD" motif in the peptide coated on the ELISA plate: this may well have caused a "background" non-FMDV specific binding. Thus possibly the QTL might be reflect variants in integrin molecules. The genes for the main integrin receptor by FMDV for entry into cells are ITGAV and ITGB6 on BTA 2 (unpub.obs) which does not appear to influence the values for the 0 and 1 time points. Nonetheless, the values seen at time 0, for both antibody and proliferation, are orders of magnitude lower than the adaptive immune responses generated to the peptide, and for the main part the time 0 values did not correlate with the responses observed at later time points.

Previous research by members of the Glass group and others have implicated the MHC locus as an important factor in determining the immune response to peptides derived from FMDV as well as protection against viral challenge [117-119]. A previous study [119], using the same population of animals indicated that MHC DRB3 alleles and polymorphisms in pocket 4 of the peptide binding cleft were significantly associated with the variation in response to the peptide. The study described here also detected QTL on BTA 23 covering the MHC locus (*BoLA*) [43] which were significantly associated with both the IgG1 and IgG2 responses across time, emphasising the importance of this locus in influencing the immune response and adding further confidence to the QTL found in the current study. However the current study has shown that even the response to a relatively simple antigen is complex and controlled by many loci.

Throughout the study the cohort of the animals was significant (the cohorts correspond to the year of birth of the animals). Environmental sources of variation were minimised as far as possible, including husbandry, food, food supplements and farm. Cohort was, however, found to be significant in the same herd for response to

a BRSV vaccine [52], a mastitis causing pathogen [133] as well as in meat and milk traits [104]. Weight and age at vaccination were also significant throughout the study. Linear regression of IgG1 AUC against age showed that the older animals had significantly higher immune responses. This suggests that even in older animals the immune system may still be developing, although possibly these changes may be hormonally related as all the animals in this study were female and there are clear gender and hormone related differences in immunity [135]. The increase in response is not a result of exposure to FMDV, unlike the situation for endemic pathogens such as BRSV which could conceivably influence the response to BRSV derived antibody. Age was also a significant factor in the IgG1 and IgG2 response to a BRSV vaccine in this experimental herd [52]. However, the animals were much younger than in the present study.

Significant breed effects have previously been shown for response to a BRSV vaccination [52], but not in response to *Staph. aureus* [133]. The current study also did not find any breed effects. It is possible that the founder breeds, Holstein and Charolais, have similar immune responses to the FMDV15 peptide, although this has not been formally tested. *Villa-Angulo et al* [136] have shown that Holstein and Charolais cattle breeds have highly correlated haplotype blocks, thus it is feasible that the similar immune response seen in this study is due to the two breeds being highly related. However, within the QTL study 6 of the 8 QTL clusters had increased phenotypes resulting from Holstein alleles, indicating that there might be breed differences in the pathways used to initiate and maintain a response to the FMDV15 peptide. Sire effects have previously been demonstrated in this herd, both in the immune response to a BRSV vaccine [52] and also to *Staph. aureus* [133]. In the present study sire effects were only significant for the IgG2 response to the FMDV15 peptide. Dam was only sporadically significant without any obvious patterns to explain the weeks it appeared significant. However, the number of calves per dam was small, so it is unlikely that enough statistical power existed to detect dam effects.

The immune response is a complex trait and across time different factors will play a role in affecting the level of a particular response e.g. the initiation of an immune response involves components of innate immunity such as macrophages and dendritic cells which signal alarm to the adaptive immune system which takes longer to respond to immunisation and invasion by pathogens. In addition, the primary response is under the influence of different factors from the secondary response, which is generally of greater magnitude and longer duration. Nonetheless, several QTL which influenced the same traits were detected across time, suggesting that there may be gene(s) that impact on both the primary and secondary responses. Thus genetic factors may underlie some of the significant correlations across time and within traits (Table 2.4). The data presented here also suggests that there are several loci which have more specific effects either at single time points or on specific immune traits, which may reflect the different cell types and factors operating at different phases of the immune response.

Inter-trait correlations were observed between the IgG1 and IgG2 responses (Table 2.3), although for the most part the QTL controlling these traits did not coincide on the same chromosomes, with the notable exceptions of the QTL on BTA 23, 95% confidence interval of which harboured the *BoLA* locus, and BTA 20. Both of these chromosomes may have genes that control both types of antibody response across time. All animals had considerably higher IgG1 than IgG2 responses, with a proportion having no detectable IgG2 response at any time point [119]. The QTL with the greatest influence on the overall IgG1 responses were BTA 4, 20, 23 and 24 which together accounted for over 25% of the variance. Apart from BTA 20 and 23, two different regions on BTA 15 and 25 were also linked to the overall IgG2 response, and these 4 regions also accounted for over 25% of the variance for overall IgG2 response. In cattle it appears that IgG1 is associated with type 2 T cell responses and IgG2 with type 1 [137]. Thus regions controlling the differing responses may harbour genes that influence the balance between Th1 and Th2 responses; plausible candidate genes under QTL include IL18 on BTA 15 and IL4R on BTA 25.

The T cell responses to the ConA mitogen and the FMDV15 peptide were also correlated, in particular the responses at the same time points. This was unexpected as immunisation with the FMDV15 peptide should not influence the responses to ConA, as the latter is a T cell mitogen. One possible explanation may be that the T cells stimulated by the FMDV peptide become more responsive to T cell mitogens; indeed proliferation was observed in the presence of medium alone and increased across time, suggesting that immunisation may have activated the overall T cell population. However, with the exception of BTA 6, 7 and 29, no co-incident QTL for T cell response to ConA and FMDV15 were observed.

Interestingly, every QTL cluster, with the exception of the cluster on BTA 25, contained at least one QTL above the 1% chromosome-wide significance level. Thus the clusters appear to highlight regions of the bovine genome that are highly associated with the immune response to the FMDV15 peptide. The two clusters containing the QTL associated with the greatest number of traits were located on BTA 20 (Table 2.3; Figure 2.4) and 23 (Table 2.3; Figure 2.5; both included in Table 2.4). The cluster located on the telomeric region of chromosome 20 has highly significant dominant effects, suggesting that over-dominance may have a role to play in these QTL. Further, this region has also been associated with a number of diseases, including resistance to *Mycobacterium avium* ssp Paratuberculosis the causative bacterium of Johne's disease [130], bovine keratoconjunctivitis (pinkeye) [55]and respiratory disease and pododermatitis (footrot) [4]. Taken together with the data presented here this suggests that gene(s) located in this region may control the response to several bacterial and viral pathogens, possibly by influencing antibody production. The current data suggest that the gene(s) underlying this QTL impact on the antibody responses across time. Further research to identify the gene(s) underlying these associations is warranted as they may represent genes for "generalised immune competence" or resistance to a broad range of pathogens.

The QTL cluster located on BTA 23 includes the bovine *MHC* (*BoLA*) region [43]. However, few markers were spaced across BTA 23, and the QTL CIs covered the whole of BTA 23. *Baxter et al* [119] recently reported that the highly polymorphic

BoLA DRB3 gene is significantly associated with the response to FMDV15 in this herd and earlier studies have indicated that *DRB3* polymorphisms are also linked to protection against viral challenge following immunisation with FMDV15 and similar peptides [118]. It is therefore likely that the causal genes underlying the part of the BTA 23 QTL effect may be coded for within the BoLA region. However, there are at least 154 predicted genes in this region [68], which may make it difficult to identify the causative mutations.

The QTL cluster on BTA 6 overlaps with a QTL that is associated with a mastitis related trait [132]. Recently *Jann et al* [138] have identified the toll like receptor (TLR) cluster of TLR1, 6 and 10 genes as the most likely candidate genes within this region on BTA 6. TLRs play an important role in both innate and adaptive immunity [139]. *Villa-Angulo et al* [136] demonstrated that BTA 6 has haplotype blocks that are similar ($r = 0.61$) between the breeds used in the current study, thus the genes in both breeds are likely to be in the same regions and orders in the cluster of TLR 1, 6 and 10.

2.5 Conclusion

QTL have been associated with a variety of traits in diseased and infected animals [129]. This study focused specifically on immune responses across time following a specific immunisation procedure to identify QTL controlling defined immune responses. The density of markers and numbers of animals used is not sufficient to fine map the QTL regions identified, however, further work is warranted especially to refine the QTL clusters, which appear to be the most promising regions. With the availability of the bovine genome sequence [68], the bovine HapMap [140] and dense bovine SNP arrays [141] unprecedented opportunities are now available to identify genes and pathways underlying immune-related traits.

Chapter 3

Quantitative trait loci associated with the immune response to a Bovine Respiratory Syncytial Virus vaccine

3.1 Background

In this chapter the same theory, methodology and animals were used as with chapter 2, except that a different immunogen, Bovine Respiratory Syncytial Disease Virus (BRSV) vaccine was used to elicit an immune response over time. A linkage analysis was completed using the antibody response to the BRSV vaccine as the phenotype. Finally, the immune responses to the two immunogens were analysed for possible correlations, and overlapping QTL.

BRSV is a pneumovirus. Like many viruses BRSV is constantly evolving, with 12 strains currently reported (www.ncbi.nlm.nih.gov/Taxonomy). The virus consists of 11 proteins with two accounting for much of its variability. The first being the most variable protein within the virus, the G (Glycoprotein) protein, which varies in size depending on which isolate is being studied, but contains a conserved region in all strains [142-143]. The G protein is also found in two forms: a membrane bound form and a secreted form, which inhibit the activation of an immune response [143]. The second protein is the F (Fusion) protein, which is also a surface protein on BRSV. The F protein is the mediator protein which aids BRSV binding to host cells [143].

The virus not only infects cattle, different strains affect sheep, goats and humans (HRSV) [144]. It is endemic in the Western world and a major cause of respiratory infections in livestock worldwide. It also causes high morbidity and thus has a high economic cost. In cattle the outcome of a BRSV infection is widely variable depending on a number of factors including age and sex [145]. In general, natural infection induces poor immunity and re-infection is common. Such is the variation within the immune response that naïve cattle can be severely affected, or, display little or no clinical signs, when infected [146]. In young cattle the virus is particularly difficult to immunise against, partially due to maternally derived antibody interfering with vaccination [144, 147-148]. O'Neill *et al* have shown a heritable component to BRSV is present, potentially allowing for breeding for increased IgG levels post BRSV vaccination.

Vaccination is a popular method of control for BRSV with both inactivated and live strain vaccines available. Administration is often via the mucosal route as this is the natural route of infection [142-143, 148]. However, there is still no consensus over which form of vaccine is best, especially in relation to vaccinating young calves, as infection is possible before the vaccination course is complete [142, 148]. Immunity to BRSV is generally considered to require neutralising antibody, but cellular immunity also plays an important role. However, cellular immunity may also induce pathology [149]. The balance between Th1 versus Th2 responses may be critical in determining the outcome of BRSV vaccination and infection, in addition, an optimum level and timing of each type of response may be required to ensure that vaccines are protective and do not predispose to disease. The Th1 cytokine, interferon-γ (IFNγ) has been associated with protection against BRSV pathology [150-152] as has the corresponding Th1 antibody isotype ,IgG2 [150]. However the Th2 associated antibody isotype, IgG1, may be required for viral clearance and protection [153]. It therefore seems likely that a balance between both Th1 and Th2 responses are required for optimal protection and reduced pathogenesis. In addition, little is known about the reservoir sites the virus uses, or how cattle eventually shed the virus [144].

Genetic loci have been shown to play a role in human susceptibility to the related pathogen, Human Respiratory Syncytial Virus (HRSV) [154], and as the epidemiology and pathology of HRSV and BRSV are similar [143], it is possible that at least a proportion of the variation in BRSV outcome, may be related to the genetically controlled response to HRSV infection. However, to date, no study of the genetic loci controlling the response to a BRSV infection has been conducted in cattle.

A previous study investigating the BRSV vaccine using the RoBoGen herd [52] showed that the vaccine induced a good immune response within the RoBoGen population, yet individual animals had highly variable levels of antibody response. *O'Neill et al* [52] went on to show that this variation was indeed influenced by genetic factors, such as sire, sex and pre-existing antibody. I have followed up the

work of *O'Neill et al* [52], and conducted a quantitative trait loci (QTL) study, to investigate the contribution of different regions of the genome to the genetic variation described in the *O'Neill et al* study. In chapter 2 and this chapter the immune responses to the BRSV vaccine and the FMDV peptide were measured using the same animals, across time. Thus I was also able discover some correlation between the traits from chapter 2 (FMDV) and this chapter (BRSV). In addition I was able to detect specific regions of the genome that appeared to control the response to BRSV vaccination as well as to the FMDV peptide.

3.2 Materials and methods

3.2.1 Acknowledgements

All statistical analysis, within this chapter, was conducted by Richard Leach. All the laboratory based assays, in this chapter, were completed by Ronan O'Neill. Immunisations were done by the Roslin Institute farm staff. John Williams' group completed the genotyping of the RoBoGen herd with the microsatellite markers.

3.2.2 Animals

The second generation of the RoBoGen herd was vaccinated with the BRSV vaccine. All male calves had unrestricted suckling with their dams, at grass, until approximately 4 months old. All female calves were weaned by 36h, segregated from the rest of the herd and raised indoors, initially on milk-replacer then weaned early onto a propriety compound diet. Any differences in the results observed between sexes were therefore compounded by differences in management.

3.2.3 Phenotypic Data

3.2.3.1 Vaccination with Rispoval and sampling

Immune response measurements were collected on 468 second generation crossbred calves (294 F2, 90 HB1 and 81 CB1). The age at first immunisation with the BRSV vaccine (Rispoval ©, Pfizer Animal Health, Surrey, UK) ranged from 88-195 days. All animals were vaccinated and IgG levels measured as described by O'Neill *et al* [52]. Each animal received 2ml of the attenuated live vaccine, intramuscularly, according to the manufacturer's recommendations. All calves were re-immunized with 2ml of the vaccine intramuscularly, 3 weeks following the initial vaccination. Blood samples were collected by jugular venipuncture: 4 and 2 weeks pre-vaccination; at week 0 (the day of first vaccination) and at 2, 5 and 7 weeks post-vaccination, providing six samples per calf. Serum was collected within 2h of sampling and stored at −20 C until testing.

3.2.3.2 ELISA for detection of BRSV-specific total IgG, IgG1 and IgG2

This assay was carried out as described in O'Neill et al, 2006 [52]. Briefly, sera were tested quantitatively by solid-phase antibody capture ELISA specific for total BRSV-IgG according to the manufacturer's guidelines (Svanovir BRSV-Ab, Svanova Biotech, Uppsala, Sweden). Samples were tested in duplicate, at a dilution of 1/25 and optical densities (OD) read at 450nm. The corrected optical density value for each sample and the control sera was calculated by subtraction of the OD value of each control antigen-coated well from that of the corresponding viral antigen-coated well and the relative optical density (ROD) value for each sample was calculated as a proportion of a positive control serum on a per-plate basis.

BRSV-IgG2 levels were tested using a modified form of the above assay. All dilutions were as described above but 100µl of 1/10,000 horse-radish peroxidase-conjugated anti-bovine IgG2 (Acris Antibodies GmBH, Hiddenhausen, Germany) was used as the secondary antibody. The corresponding ROD values were also calculated as above. To obtain the IgG1 levels, IgG2 was subtracted from the total IgG.

Area under the curve (AUC) for each trait was also calculated (using the trapezoidal rule [35]) to provide a single trait that reflected the overall response of each animal (explained further in section 2.2.5).

3.2.3.3 Immunisation with the FMDV15 peptide

Please see section 2.2.3

3.2.4 Statistical Analysis

The IgG concentrations were $\log_{10}$ transformed to obtain a normal distribution and constant variance, allowing for REML analysis.

Significant factors, such as sire, within this study have been previously calculated by O'Neill *et al* [52] . For the QTL calculation, the final model included sire and dam as

random effects; the fixed effects within the model, with appropriate degrees of freedom (df), were *line* (F2, CB1, HB1; 2d.f), *age* (age at vaccination), *cohort* (1, 2, 3; 2d.f) and *calculated weight* (weight of the animal at first vaccination date calculated from regression of animal weight at other time points pre and post vaccination).

Thus the model was:

$Y_{ijk}= \mu + L+C+S+\beta_1.m+\beta_2.a+u_j+g_k+e_{ijk}$

Where: Y_{ijk} is the observed value of the phenotypic trait; μ, population mean; L, the fixed effect of line (3 Lines); C, the fixed effect of cohort (3 Cohorts); S, the fixed effect of sex (male or female); β_1, the linear regression on the covariate of age at vaccination m (m = d469-d609); β_2, the linear regression on the covariate of weight a (a= 361-744kg); u_j, the random effect of sire; g_k, the random effect of dam g; e_{ijk}, the residual error $e \sim N(0,I\sigma^2_p)$. The residual variance from the model was used to calculate correlations.

3.2.5 Correlations

The residuals, for each time point, from the REML models were saved and used to calculate all the correlations between the immune responses at differing time points throughout the study. With the exception of sex, the same REML models were as used in the previous FMDV study [155].

A direct comparison of the IgG levels in response to the FMDV15 peptide could not be made with the IgG levels in response to the BRSV vaccine, as they were measured in differing units. Thus a Spearman's rank correlation, using the residuals of the REML models, was used to calculate the correlations between the responses to the FMDV15 peptide and to the BRSV vaccine for animals that were tested for both traits (i.e. the 195 second generation females immunised with the FMDV peptide and vaccinated with BRSV vaccine).

3.2.6 Genetic Markers

Please see section 2.2.6

3.2.7 QTL Analysis

Please see 2.2.7

3.2.8 Refining QTL

Please see 2.2.8

3.3 Results

A total of 468 second generation (245 male and 223 female) animals of the RoBoGen Charolais X Holstein herd were phenotyped for total IgG, IgG1 and IgG2 responses to a BRSV vaccine across time. The whole herd (984 animals) was genotyped with 165 microsatellite markers. The female heifers were both vaccinated with the BRSV vaccine and immunised with the FMDV peptide and the data from both studies were compared.

3.3.1 Kinetics of vaccination

Essentially all of the parameters of all the traits in this study have been previously described (for BRSV [52] and for FMDV [155]). A summary table of the means, ranges and standard deviations of the traits are presented here for clarity (Appendix 3.1). Considerable variation between animals was apparent, although all animals made a significant response to both immunogens. In both studies, the IgG2 response was lower than the IgG1 response.

All traits were analysed for possible correlations with each other and many significant correlations within specific traits were found across time (Appendix 3.2). The FMDV traits have been described previously [155] and similar results were found for the BRSV traits, in that the IgG1 and IgG2 responses to BRSV vaccination at earlier time points correlated with their next corresponding time points. Thus the IgG1 level at week 2 correlated with the IgG1 level at week 5 ($r=0.26$, $p<0.01$) and the IgG1 level at week 5 correlated with the IgG1 level at week 7 ($r=0.69$, $p<0.01$). Similar correlations were found for the IgG2 levels. Furthermore, each time point correlated with the AUC for each respective isotype level, with the highest correlation found between the IgG2 week 5 post vaccination and the IgG2 AUC measurement ($r=0.79$, $p<0.01$). The two BRSV specific IgG isotype levels were also significantly positively correlated with each other (Appendix 3.2). The highest correlation was between IgG1 and IgG2 at week 7 ($r=0.47$, $p<0.01$). As expected, there were also negative correlations between the levels of antibody at week 0 and

levels post-vaccination as the animals had pre-existing levels of IgG derived from colostrum, which inhibited the vaccine response [156-157].

Correlations between the responses to BRSV vaccination and the FMDV15 peptide immunisations were analysed using Spearman's rank correlations. It should be noted that the two immunisation schedules were carried out at completely different times, when animals were of different ages and thus they were unlikely to interact with each other. Few significant correlations were found between the responses to FMDV and BRSV, and these were generally low (Appendix 3.2). There was some evidence that the IgG2 isotype responses to both immunogens may have a relationship in that the FMDV specific IgG2 response at week 8 was correlated with the BRSV specific IgG2 response at week 5 ($r=0.2$, $p<0.01$) and the FMDV IgG2 and the BRSV IgG2 AUC measurements also correlated, with an $r=0.15$ ($p<0.05$).

3.3.2 QTL Results

A total of 27 QTL for BRSV response were discovered across 13 autosomes, with 9 QTL found on the initial genome scan and 4 QTL discovered when background effects were taken it account (Table 3.1). Seven QTL were above the 1% chromosome level, and of these, one QTL on BTA 17 was at the 5% genome wide significance level, explaining 3.47% of the phenotypic variance (σ_p) (Figure 3.1). The remaining 16 QTL were all above the 5% chromosome wide significance level.

Of the 27 QTL detected, 16 were associated with the IgG2 response, whilst 7 QTL were associated with the total IgG response and only 4 with the IgG1 response. Thus loci accounting for a greater proportion of genetic effects controlling the phenotypic variance of the IgG2 antibody levels were identified in comparison to the total IgG and IgG1 antibody levels. However the traits are linked (as IgG1 and IgG2 are components of total IgG), and one QTL that spans time maybe counted multiple times, thus if multiple QTL have the same flanking markers upon a chromosome it is possible that they are the same QTL. If the 27 QTL are again summed taking this into account then there appears to be 17 individual QTL that exist independently of

time and trait (1 on BTA2; 2 on BTA3; 2 on BTA7; 1 on BTA8, BTA9, BTA10, BTA14 and BTA15; 2 on BTA17 and BTA18; 1 on BTA23, BTA24 and BTA28).

Chromosome[1]	Trait[2]	cM[3]	F[4]	a[5]	d[5]	Var%[6]
2	Total_IgG_week_0	18cM	5.47	-3.67**	6.22**	2.11
2	IgG2_minus_week_2	19cM	6.25	-4.22*	9.07**	2.67
3	IgG2_minus_week_2	37cM	5.19	1.89	6.77**	2.24
3	IgG2_week_2	98cM	4.88	1.03**	0.35	2.12
7	IgG2_week_0	69cM	5.21	0.74**	-0.1083	2.26
7	Total_IgG_week_2	22cM	4.92	3.97*	-4.8454	2.14
8	IgG1_AUC	38cM	5.9	26.36***	-3.34	2.52
8	IgG1_week_7	44cM	5.63	4.77***	0.32	2.42
8	Total_IgG_AUC	43cM	4.98	25.80**	-3.62	2.12
8	Total_IgG_week_7	47cM	5.53	5.63***	0.7997	2.40
9	IgG2_week_2	45cM	5.47	-0.87*	0.87	2.37
10	IgG2_week_0	53cM	7.01*	-0.07	-1.03***	3.01
14	IgG2_AUC	15cM	6.82*	13.67***	5.77	2.92
14	IgG2_week_2	23cM	5.79	0.99**	1.19*	2.51
14	IgG2_week_7	16cM	5.82	4.14***	-0.13	2.51
15	Total_IgG_week_0	60cM	4.85	3.64**	-2.73	2.10
17	IgG2_minus_week_2	15cM	4.88	-6.13	6.83	2.09
17	IgG2_minus_week_2	57cM	4.92	-6.44**	3.51	2.10
17	IgG1_week_2	76cM	8.17**	-4.30***	1.99	3.47
17	Total_IgG_week_2	76cM	6.67*	-4.20***	1.51	2.87
18	IgG2_week_2	19cM	7.29*	-3.58***	-2.20*	3.15
18	IgG2_week_2	38cM	5.81*	-0.96**	-0.90	2.52
18	Total_IgG_week_7	18cM	5.27	-4.67**	-1.83	2.28
23	IgG2_AUC	27cM	4.97	17.17**	1.71	2.15
23	IgG2_week_2	31cM	6.82*	2.03***	0.36	2.86
24	IgG2_week_2	11cM	4.15	0.16	1.48**	1.81
28	IgG1_week_7	0cM	4.37	3.41*	0.64	1.89

Table 3.1: All QTL located in this study

1. Chromosome: the chromosome each QTL is located on.
2. Trait: each trait is shown as follows: total IgG or IgG isotype response, followed by the week relative to vaccination.
3. cM: the QTL position on the chromosome (centiMorgans).
4. F: the F-statistic of each QTL, all are at least 5% chromosome wide, * 1% Chromosome wide and ** 5% Genome wide.
5. "a" and "d" are the additive and dominance effects of each QTL, *=p<5%, **= p<1% and *** = p<0.01%.
6. Var%: the additive phenotypic variance explained by the QTL.

Nearly all of the QTL (23) identified showed significant additive effects ($p<0.05$), whereas only 7 showed significant dominant effects ($p<0.05$). Both Holstein and Charolais derived alleles were shown to have additive and/or dominance effects on

QTL. Fourteen of the 27 QTL with significant additive effects had the Holstein derived alleles increasing the trait value (4 total IgG QTL, 2 IgG1 QTL and 8 IgG2 QTL); the other 9 had Charolais derived alleles increasing the trait value (3 total IgG QTL, 1 IgG1 QTL and 6 IgG2 QTL) as shown in Table 3.1. Seven QTL had significant dominance effects, and of these 3 QTL had dominance effects with increases of the trait attributed to the Holstein alleles (all IgG2 QTL), whilst 2 QTL were traceable to Charolais alleles increasing the trait (Total IgG QTL and one IgG2 QTL). Overall, there did not appear to be a breed bias towards additive or dominance effects. However, the alleles derived from the Holstein breed appeared to increase the immune traits more frequently than the Charolais alleles in this study.

The QTL associated with total IgG levels tended to overlap (+/- 7cM of the peaks) with either IgG1 or IgG2 QTL. Five of the seven total IgG QTL overlapped (BTA2, two on BTA8, BTA17 and BTA18; Table 3.1), reflecting the fact that the total IgG phenotype was made up from the components of the IgG1 and IgG2 phenotypes. These overlapping QTL also included an overlap between QTL associated with total IgG AUC and the IgG1 AUC on BTA 8 (Figure 3.2).

A total of 8 QTL were associated with the IgG levels prior to vaccination, whereas the majority of QTL were associated with the response post vaccination and were mostly on different chromosomes from those associated with the pre-vaccination levels (Table 3.1). The trait with the greatest number of different QTL associated with it was the IgG2 response 2 weeks post vaccination, with a total of 7 QTL, in contrast to the single QTL associated with the IgG1 response at the same time point. Fewer QTL were associated with the antibody response following the vaccine boost, with only one associated with the IgG2 response at week 7, but two for the IgG1 response at this time point.

The most significant QTL were mainly associated with the IgG2 responses, with 5 of the 7 QTL above the 1% chromosome significance level (including the QTL also at the 5% genome wide significance level). These QTL also had other QTL within the confidence intervals for differing traits and time points (with the exception of the

QTL located on BTA 10, Table 3.1). For example: BTA 14 contains a QTL associated with the IgG2 AUC measurement at the 1% chromosome wide significance level (Figure 3.3) and within 8cM of the peak of this QTL are QTL for IgG2 week 2 and IgG2 week 7 post vaccination. In addition, another 2 QTL associated with the immune response post vaccination (IgG2 week 2, 1% chromosome wide and the IgG2 AUC measurement, 5% chromosome wide) were discovered on BTA 23, with their peaks separated by 5cM (Figure 3.4).

Fourteen of the QTL identified in this study were within the confidence intervals of QTL detected in the FMDV study [155]. Further, the peaks of four of these QTL were within 10cM of QTL peaks associated with the response to the FMDV peptide and included BTA 9 at 45cM, BTA23 at 27cM and 31cM and BTA24 at11cM. Of the overlapping QTL, the QTL on BTA 23 were the most striking, as two QTL from both studies overlapped across the same regions of BTA23 (Figure 3.4) which contains the MHC region.

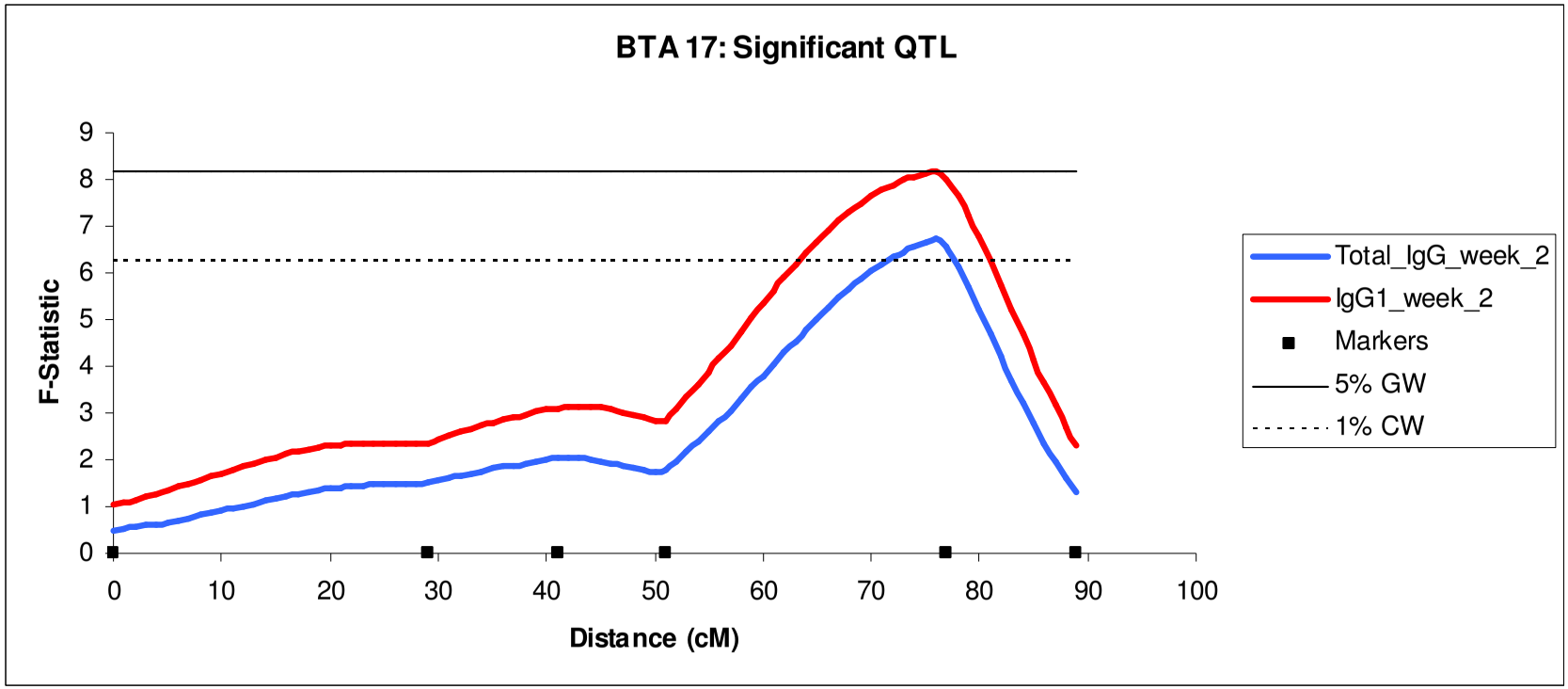


Figure 3.1: BTA 17: Significant QTL.
F-statistic profiles for the total IgG and IgG1 response elicited by the BRSV vaccine two weeks post vaccination. The dashed horizontal line represents the threshold of the 1% chromosome wide significance level ($F = 6.26$) and the constant horizontal line represents the threshold of the 1% chromosomal wide significance level ($F = 8.16$).

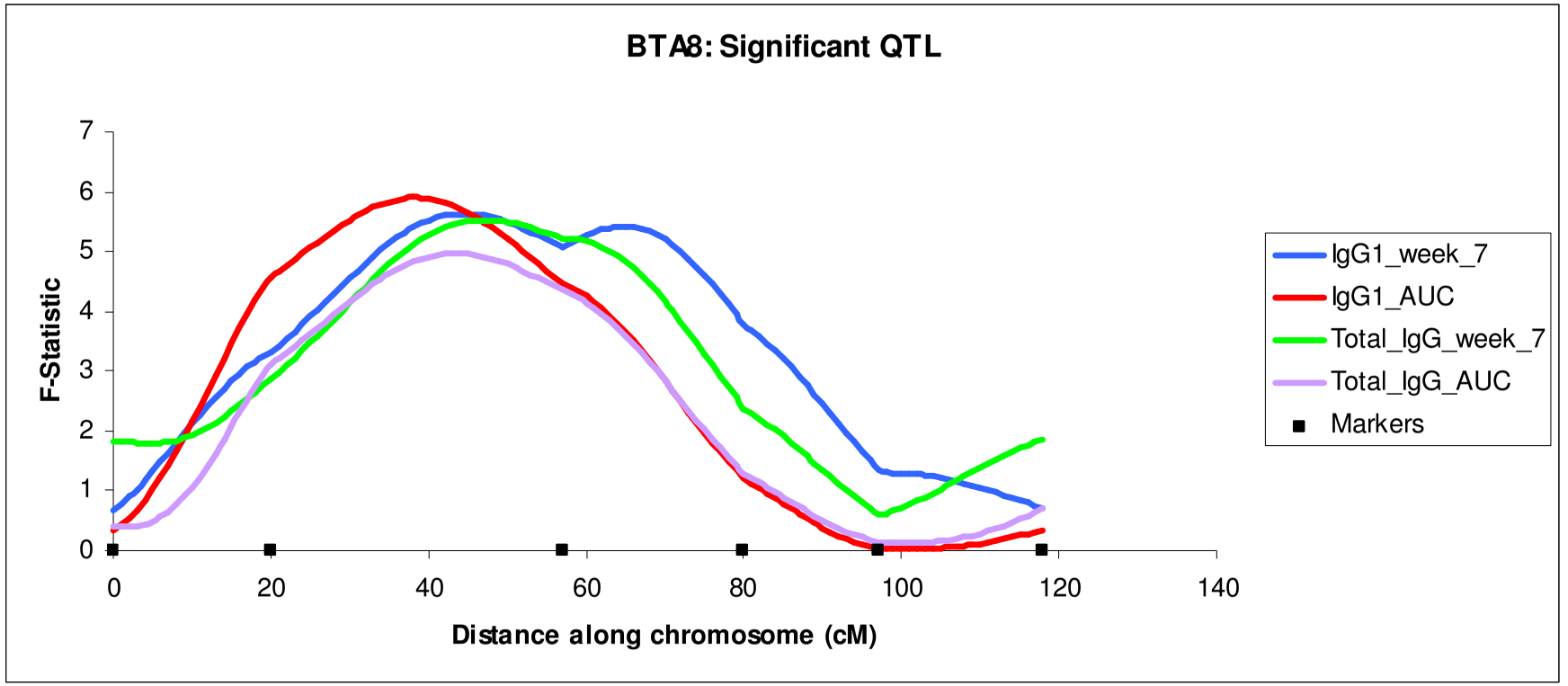


Figure 3.2: BTA 8: Significant QTL
F-statistic profiles for the total IgG and IgG1 responses elicited by the BRSV vaccine seven weeks post vaccination and using the AUC measurement. All the QTL are above the 5% chromosome wide significance level.

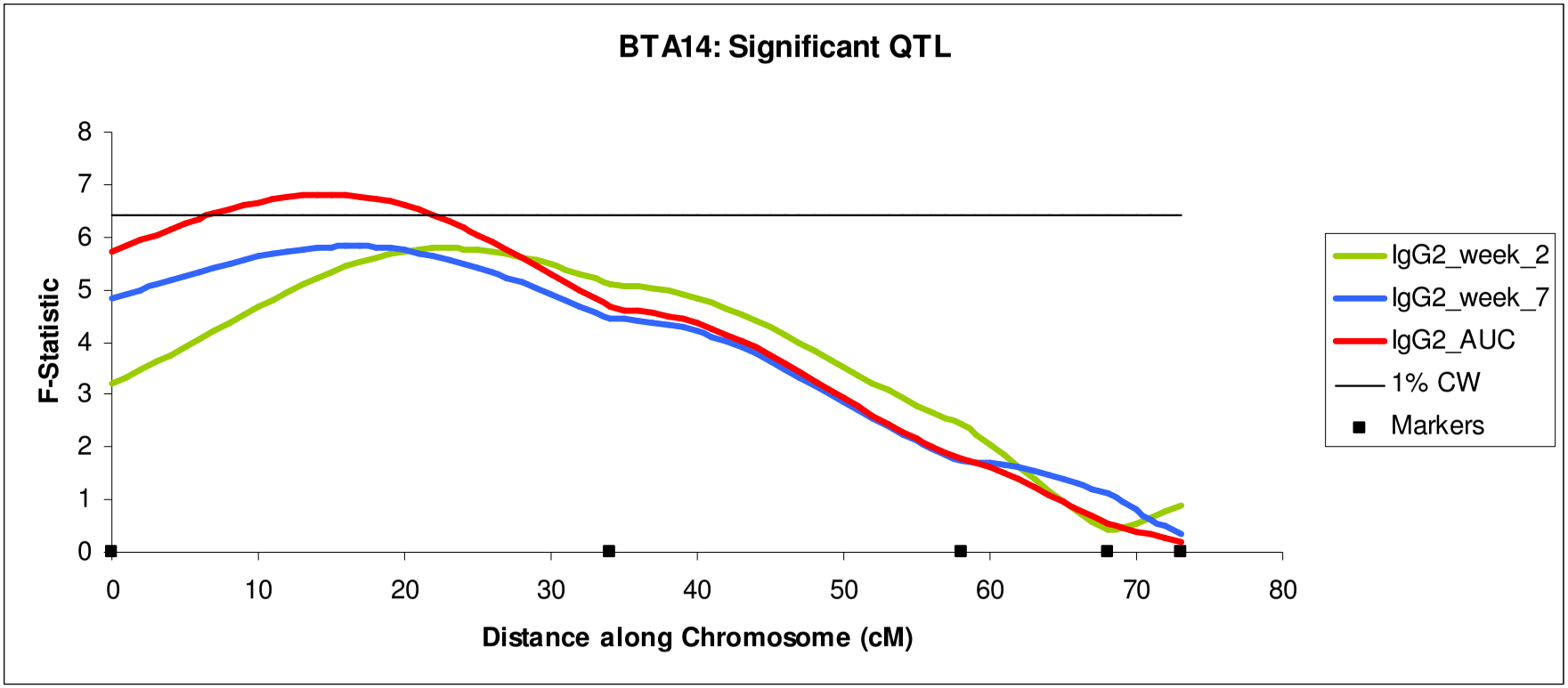


Figure 3.3: BTA 14: Significant QTL
F-statistic profiles for the IgG2 response elicited by the BRSV vaccine two and seven weeks post vaccination and using the AUC measurement. The constant horizontal line represents the threshold of the 1% chromosomal wide significance level ($F = 6.43$).

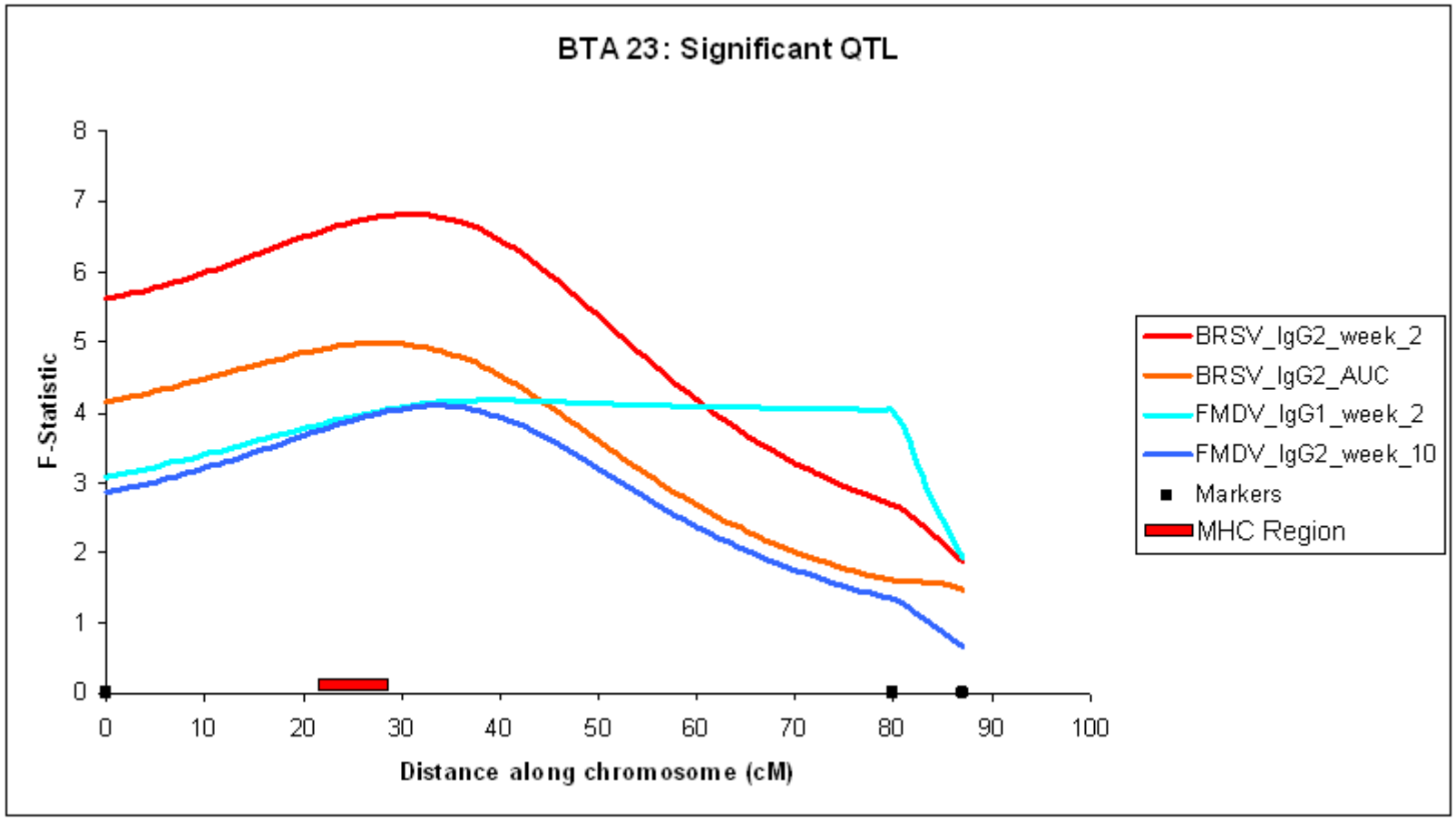


Figure 3.4: BTA 23: Significant QTL
F-statistic profiles for the IgG2 response elicited by the BRSV vaccine two weeks post vaccination (peak, 31; F-statistic 6.82) and the AUC measurement (peak, 27; F-statistic, 4.97) and for the IgG1 (peak, 39; F-statistic, 4.16) and IgG2 (peak, 33; F-statistic, 4.08) levels elicited by the FMDV peptide 2 and 10 weeks post immunisation, respectively. The red rectangle on the x-axis shows the approximate location of the MHC region.

3.4 Discussion

In this study I have discovered a number of distinct QTL which control the immune response to BRSV vaccination as well as clearance of maternal antibody in cattle. Although there was evidence for QTL within the MHC region on BTA23, the majority of the QTL described in this paper do not lie within this region. The results suggest that some chromosomal regions may control both the IgG1 and IgG2 responses, however, most of the QTL detected appear to play a role in the IgG2 response. This study is a follow on from a previous study of the same crossbred herd which found strong heritability (h^2 =0.52 with respect to total IgG levels over time (day0-day35)) and significant sire effects in the IgG response post vaccination [52]. In the present study 27 significant QTL were associated with BRSV specific IgG1, IgG2 and total IgG levels, pre- and post-vaccination, which were located on 13 bovine autosomes. The multigenic nature of the response must at least in part, reflect the complexity of host immunity.

In the previous study the Holstein backcross calves were shown to exhibit significantly higher antibody levels than the Charolais backcross calves pre-vaccination but not post-vaccination [52]. The QTL found in the current study did not reflect this, as both breeds had similar QTL additive effects, although the Holstein backcrosses generally had higher antibody levels pre-vaccination. This is in contrast to a study which investigated bovine keratoconjunctivitis [4], where the additive effects of QTL originated from a breed with known low prevalence of the disease [158]. This suggests that further QTL are yet to be discovered, which may explain the higher antibody levels in the Holstein backcross cattle pre-vaccination. Furthermore, most of the significant dominance effects of QTL associated with pre-vaccination levels, were derived from the Charolais breed, suggesting a complex mode of inheritance may be responsible for the observed phenotypes, such as parent-of-origin effects [159] or over-dominance [160]. Further studies would be warranted to investigate these dominance effects as breeding programs only utilise additive effects and not dominance effects [161].

Although a considerable number of QTL were discovered in this study, there were fewer QTL than reported for the response to an FMDV peptide in the same RoBoGen herd [25] (Chapter 2). One reason for this may have been that in the current study the antibody levels were measured by a commercial kit intended for epidemiological surveys of prevalence and the sera were used at a fixed dilution (1/25). Whereas sera used in the FMDV ELISAs were titrated to identify the optimum dilution [25]. Thus it is possible that variation in IgG level in high titre samples, that were above the maximum O.D for the assay for anti BRSV antibody, would not have been detected.

The persistence of circulating maternal antibodies has been shown to have an effect on the efficacy of vaccination against several important infectious diseases [162]. It is considered preferable to vaccinate calves against BRSV at an early age, because it is endemic, even though maternal antibodies affect BRSV vaccine efficacy [163]. In the present study the levels of anti-BRSV IgG were declining prior to vaccination, which is most likely due to the reduction in the levels of circulating maternal antibody [52, 157]. Thus the detection of QTL for antibody response post-vaccination may have also been affected by the unknown effect of maternal antibody on the efficiency of immunisation.

Eight QTL were detected associated with the BRSV-specific antibody levels pre-vaccination. Two QTL on BTA 10 and 15, were not associated with the response post-vaccination, while the others were on chromosomes with post-vaccination QTL, but with apparently different peaks. These QTL may therefore reflect genes/pathways which are responsible for regulating the levels of maternal antibody. The transfer of IgG from dam to calf via colostrum has high heritability [164] and results presented here suggest that IgG transfer may be partly controlled by the genetics of the calf. Furthermore, the neonatal Fc receptor, a heterodimer consisting of a MHC class I α-chain homolog located on BTA18 [165] and beta-2-microglobulin (b2m), located on chromosome 10 [166], may play a significant role in regulating the level of maternal antibody [165]. Polymorphisms in the MHC α-chain homolog on BTA 18 have been associated with the levels of IgG in newborn

calves [165]. However the QTL detected on BTA18 were associated with the post-vaccination response and not pre-vaccination levels. Furthermore, the gene for the MHC α-chain homolog is located outside of the final marker on that chromosome, and thus outside the confidence intervals for these QTL; further genotyping in this region may be able to provide better resolution. In addition, the gene encoding b2m, which has been associated with the failure of transfer of maternal IgG to calves [167] is positioned just outside of the confidence interval of the pre-vaccination QTL located on BTA10 which was the only QTL detected in the current study on this chromosome. Addition of markers to the analysis of BTA 10 may better define the QTL position and determine whether b2m is a putative candidate gene controlling pre-immune IgG. Identifying the genes underlying the QTL associated with variation in levels of maternal antibody could potentially aid the design of vaccines which reduce or avoid the effects of maternal antibodies.

A single QTL was identified, located on BTA 14, which influenced the response at different time points, which appears to influence the IgG2 response at 2 and 7 weeks as well as the overall (AUC) IgG2 response post vaccination. This is in marked contrast to the 8 clusters of QTL that were associated with the response to the FMDV peptide across time [155]. However more time points were measured in the FMDV study which may have increased the chances of detecting QTL across time. Also the FMDV antibody levels were measured with greater accuracy and were not influenced by maternal antibody, both of which may have reduced the ability to detect BRSV related QTL in the current study. Nonetheless the data indicate that distinct genes operate early in the BRSV vaccine response compared to those that underlie variation following the boost. The QTL associated with the IgG2 response at week 2 following the first immunisation also accounted for the most phenotypic variance. In addition, the IgG2 response at week 2 had the most QTL associated to it within this study, which altogether explained over 17% of the IgG2 response at this time point. As the responses at later time points also correlate with the week 2 response, this suggests that these QTL may be particularly important in determining the overall IgG2 response. In contrast, no QTL were identified which were associated with either IgG isotype, 5 weeks post vaccination. Previously, it has been shown that the

variation in IgG1 and IgG2 at this time point is small among the different second generation classes (F2 CB1 and HB1) [157]. Understanding the genetic control of the variation in the IgG2 response (associated with Th1 responses) at early time points, may suggest new ways to modulate the Th1 and Th2 balance induced by vaccination, which may be relevant for improved protection and avoidance of adverse pathology following vaccination [168]. Furthermore markers associated with these genes would be valuable for breeding for enhanced disease resistance.

In contrast to the response to the FMDV peptide [155] (shown in Chapter 2), the anti BRSV IgG1 and IgG2 levels were highly correlated, both pre and post vaccination,, however, no QTL for IgG1 levels were identified that overlapped with QTL for IgG2 levels, which is in contrast to the QTL for FMDV peptide response where three chromosomal regions on BTA 20, 23 and 25 had QTL peaks for both antiFMDV peptide IgG1 and IgG2 responses [155] (Chapter 2). These findings imply strong correlation seen at the phenotypic level does not necessarily indicate that the phenotypes share genetic control. Thus although the levels of both antibody isotypes for BRSV increased at similar rates post-vaccination, different genes may be involved in controlling the response of IgG1 and IgG2. If so, these genes may explain, at least in part, how a balance between Th1 and Th2 is maintained [153], by animals which show little pathology post infection, potentially enabling the markers discovered here to be included in marker assisted selection for reduced pathology of BRSV.

Almost half of the 27 QTL significantly associated with IgG levels pre and post BRSV vaccination, lie within the 90% confidence intervals of the QTL associated with antibody responses to the FMDV peptide [155] (Chapter 2). Indeed, 10 of these QTL are associated with the same IgG isotype, suggesting that the same genes influence IgG levels post immunisation with different immunogens. Furthermore, QTL identified in the current study also fall within the confidence intervals of QTL associated with other immune traits, including: the change of somatic cell score in response to Mastitis [169] on BTA15; antibody response to *Mycobacterium avium ssp. paratuberculosis* [170] on BTA 8 and 9; and bovine spongiform encephalopathy

infection [171] on BTA 17. This raises the possibility that the immune responses to different stimuli may be controlled, to some degree, by similar pathways, even if the initial detection of the infection occurs through distinct mechanisms, downstream signalling may converge on similar pathways. If the genes underlying these QTL are involved in pathways that control responses to a wide range of pathogens they may be suitable for selective breeding for disease resistance in general. In addition, understanding of the underlying genes and pathways could point to new targets for future adjuvants.

The MHC locus is expected to be a significant component underlying variation in immune response [6], as it contains many polymorphic and immune-related loci [43, 68]. Indeed in both the current study and the FMDV peptide study [155] (Chapter 2) QTL were discovered that span the MHC locus. Indeed, earlier studies have shown that polymorphisms at the *DRB3* locus are associated with response to BRSV [118, 172] as well as protection against viral challenge following immunisation with the FMDV peptide and similar peptides [119]. However, the two QTL that span the MHC loci that were identified in the present study only account for 3% of the phenotypic variation, which together with previous data from the FMDV study [155] (Chapter 2), indicates that many other regions of the genome are involved in controlling variation in immune responses.

The human strain of respiratory syncytial virus (HRSV), which is closely related to BRSV, is a major cause of respiratory disease in young children, where the epidemiology and pathogenesis of infection is similar to that of BRSV [173]. The severity of disease in young children caused by HRSV is significantly associated with genes expressed during both innate and acquired immune responses [174]. Eight of the twenty one genes implicated in the human response to HRSV [174], have orthologs within the QTL in the current study, on BTA 2, 3, 15, 17 and 23. These are STAT1 (BTA 2), TNF (BTA 23), VCAM1 (BTA 3) and IL15 (BTA17) which play roles in innate immunity and CD28 (BTA 2), STAT1, IL17 (BTA23) which play roles in adaptive immunity [175]. The QTL with the highest significance in the current study (on BTA 17) encompassed IL17 within its 95% confidence intervals.

Two further genes, from the *Janssen et al* study [174], within these QTL confidence intervals (MS4A2; FCER1A, both located on BTA 15) are associated with asthma allergies as well as severe RSV disease. It has also been suggested that a Th2 biased response in cattle, associated with induction of IgE, is correlated with pathology [176], and thus polymorphisms in these genes may also influence the pathology observed in young cattle infected with BRSV. It is interesting to note that some of the SNPs within IL15, STAT1, VCAM1 and TNF associated with HRSV in the human study [174] are also polymorphic in the bovine genome, making them very good candidates for further study.

3.5 Conclusion

This is the first time that the natural variation in the clearance of maternal antibody and the immune response to a BRSV vaccine has been successfully exploited to locate significant QTL in distinct regions of the bovine genome. A greater number of QTL were found associated with IgG2 response, and these explained a larger part of the genetic variation, than were found for IgG1 response. Some of the QTL identified overlap with QTL associated with the response to a FMDV peptide (Chapter 2). Research is ongoing to further reduce the QTL confidence intervals, with the aim of identifying the underlying gene variants. This information may be used to breed for disease resistance and the production of more efficacious vaccines through the discovery of pivotal genes and gene pathways.

Chapter 4

Fine mapping genome regions significantly associated with the immune response following immunisation

4.1 Introduction

Chapter 4 uses the same phenotypes defined in chapter 2 (FMDV peptide responses) and chapter 3 (BRSV vaccine responses). In addition an extra phenotype is used, the interferon gamma (IFN γ) levels following immunisation with the FMDV peptide at weeks 0, 4 and 10. The AS methodology used in this chapter makes use of population-wide LD, rather than familial LD that LM uses, to detect SNP that are significantly associated with a trait, thus making fine mapping QTL within the RoBoGen herd possible.

The results from chapters two and three in this thesis have shown that multiple regions of the bovine genome play a role in the antibody response to a peptide derived from a Foot-and-Mouth Disease Virus (FMDV) [155], as well as the antibody response to a vaccine against Bovine Respiratory Syncytial Virus (BRSV) [177]. In addition, a number of coincident QTL occurred in both chapters, suggesting that there may be underlying genes that control the immune response to multiple pathogens. However, the quantitative trait loci (QTL) spanned relatively large regions, making it impossible to highlight any candidate genes or causal polymorphisms with any certainty.

Difficulties in identifying causal polymorphisms are partly because complex traits, such as the immune response, reflect the outcome of many processes. In livestock species, two main approaches to identifying gene variants controlling complex traits have been employed: candidate gene studies and whole genome scans. The former are dependent on prior knowledge of the main pathways involved in the trait whereas a whole genome scan makes no prior assumptions. Both approaches have limitations as generally there are many candidate genes, and the availability of markers and genetic architecture of the population being surveyed restricts the precision with which the causal polymorphisms can be identified. Within this chapter, a limited number of SNP were genotyped in the RoBoGen herd using two different methods which used the principles of both candidate gene studies and whole genome studies to attempt to refine QTL from chapters 2 and 3 and detect novel significant SNP

associations. The first method selected SNP that were in candidate genes such as the Toll-like receptors (TLRs) and their down stream signalling partners. Whereas the second method selected SNP, with equal distances between them, that were located within the QTL confidence intervals of chapters 2 and 3. These methods aimed to select markers which were most likely to present significant results.

The first method selected SNP in TLRs as they are strong candidates for disease resistance traits as well as targets for vaccine adjuvants [178]. The host employs Pathogen Recognition Receptors (PRRs), such as TLRs, to detect the initial invasion of pathogens and signal alarm. The Glass laboratory have previously identified a number of non-synonymous and positively selected SNP in bovine TLRs which may have functional consequences [179]. In addition, QTL associated with the immune response to the FMDV peptide were located on BTA6, which contains the TLR1, 6 and 10 cluster. Furthermore, many TLRs and their down-stream signalling partners have been identified as strong candidates within QTL associated with disease resistance traits across multiple species [138], strongly suggesting that TLRs indeed play a pivotal role during an immune response.

In the second approach SNP were selected which fell within the confidence intervals of the QTL discovered in chapters 2 and 3. The phenotypes measured in response to the FMDV peptide and BRSV vaccine would likely use the B or T cell pathways. If genes within these pathways were within the confidence intervals of previously detected QTL, then SNP would also be selected and genotyped in these genes. This approach aimed to capitalise on regions of the genome which had already been significantly associated with the immune responses in previous chapters. As a result, the SNP found to be significant in these regions would refine previous QTL and possibly highlight genes in which SNP were in LD with causal polymorphisms.

An Association Study was conducted using phenotypes that were considered relevant to protection against FMD and BRSV. A panel composed of 183 SNP markers located within some of the identified QTL regions [138, 155, 177], as well as within the ten TLRs and their down-stream signalling partners [138, 180] aimed to increase

the power with which QTL could be detected. The results of this chapter show that significant SNP were located in genes with known biological relevance to an immune response, including TLR4 and TLR8 as well as in novel genes not previously associated with variation in immune responsiveness. This study may lead to the identification of selectable markers for increased disease resistance, and also may suggest new targets for the design of vaccines that enhance the host immune response to pathogens in all individuals regardless of genotype.

4.2 Materials and Methods

4.2.1 Acknowledgements

Richard Leach completed all statistical work in this chapter, with the exception of the PAML section (4.2.9), which was conducted by Oliver Jann. In addition, Oliver Jann also compiled a table of SNP located in TLR genes.

4.2.2 Animals

Please see sections 2.2.2 and 3.2.2 for details about the FMDV and BRSV animals, respectively

138 RoBoGen animals had IFNγ levels measured (98 from the F2, 25 from the HB1 and 15 from the CB1).

4.2.3 Immunisation and sampling

4.2.3.1 FMDV15 peptide

Please see section 2.2.3

For the T cell and IFN-γ analysis blood was collected aseptically into heparin tubes.

4.2.3.2 BRSV vaccine

Please see section 3.2.3.1

4.2.4 Phenotype measurement

4.2.4.1 Whole blood T-cell Proliferation assay for the FMDV peptide

Please see section 2.2.4.1

4.2.4.2 ELISA for detection of FMDV15-specific IgG1and IgG2

Please see section 2.2.4.2

4.2.4.3 IFN- γ Assay

A Biosource Bovine IFN-γ Enzyme Amplified Sensitivity Immuno Assay (EASIA) kit (Catalogue number KAC123, Biosource International, now Invitrogen) was used to detect the IFN-γ levels essentially according to the manufacturer's guidelines, except that FMDV peptide and ConA were used as the test antigens, and ovine recombinant IFN-γ (a kind gift from Professor Gary Entrican, Moredun Research Institute, UK) was used to determine IFN-γ concentrations. Duplicate whole blood samples were incubated as described above for the T cell assay, except that the final concentrations were: 2.5μg Con A/ml and 1ug FMDV peptide/ml and the samples were centrifuged after 24 hours, at 2000rpm for 10 minutes and supernatants collected and stored at -20 ^{0}C until they were tested. Initially, 100μl of the samples and IFN-γ standards (4000, 3000, 2000, 1000, 500, 100pg/ml diluted as described above for the samples), were incubated with 50μl of "Incubation Buffer" (Biosource kit) in 96-well strips coated with a capture monoclonal antibody to bovine IFN-γ (which cross-reacts with ovine IFN- γ) at room temperature (RT) for 60 minutes on a shaker, before being washed 3 times with 400μl of "Working Washing Solution" (Biosource kit). 100μl of an anti-bovine IFN-γ horse radish peroxidise (HRP) conjugate was added to each well, and again incubated at RT for 60 minutes, on a shaker. The plates were washed again and then 100μl of TMB was added to each well and all samples were incubated at RT for 15 minutes on a shaker. Finally 200μl of stop solution was added and the optical density of each well was read at 450nm on a Victor^{2} 1420 Multilabel counter (Wallac, now PerkinElmer). Optical density (OD) values were converted into concentrations using a standard curve derived from dilutions of ovine IFN-γ using a simple linear regression (Genstat) and the results were expressed in pg/ml. The intra- and inter-plate replicates were high with r^2 values of at least 0.80. The negative control values for the IFN-γ levels were then subtracted from the results obtained for FMDV15 and ConA and these values were used to convert the IFN-γ levels to a binomial trait (see statistical tests below).

4.2.4.4 ELISA for detection of BRSV-specific total IgG and IgG2

Please see section 3.2.3.2

4.2.5 Genetic Markers for the Genotyping Panel

All SNP markers for the genotyping panel were sourced from Ensembl [181]. Markers were placed in three ways (Appendix 4.1). Firstly, a program script (Perl [182]) was written to select bovine SNPs for genotyping using a Perl-Ensembl Application Programming Interface (API) (http://www.ensembl.org/info/docs/api/index.html). SNP were selected if they fell within the 95% confidence intervals of QTL from our previous studies [155, 177] (Appendix 4.4 and route 1 Appendix 4.1). Antibody levels and T cell proliferation were measured as phenotypes, thus a list of genes from the B-cell and T-cell pathways were downloaded from the KEGG database (http://www.genome.jp/kegg/). If these genes were located within the 95% confidence intervals of the QTL, then SNPs within the exons of these genes were preferentially chosen for the genotyping panel. In addition, a set of 31 SNPs on BTA 6 derived from a previous study investigating meat quality (Gutierrez-Gil et al, manuscript in prep), were also included (Appendix 4.3, SNP without "rs" numbers) as they fell between the confidence intervals of QTL in the FMDV study [155].

The other two methods of selected non-synonymous SNPs within the TLR genes (TLR1 – TLR10) (route 2 in Appendix 4.1), and their down-stream signalling partners and in other genes identified as within the syntenic blocks identifying cross-species QTL for disease resistance [138, 179-180] (route 3 in Appendix 4.1), in a candidate gene approach (Appendix 4.5).

A total of 425 selected SNPs were genotyped using an Illumina BeadArray platform (http://www.illumina.com/technology/goldengate_genotyping_assay.ilmn, Appendix 4.3). Genotyping was performed by ARK genomics (http://www.ark-genomics.org) with at least 5µl of DNA (50ng/µl) per sample from each phenotyped animal.

Each marker was tested for quality to ensure reliable results. To pass quality control each SNP had to have: a minor allele frequency above 5%, a call rate of above 95% and be within Hardy-Weinberg equilibrium (FDR<0.2). The SNP alleles were then recoded as 0, 1, 2 with 0 =AA, 1=AB and 2=BB, to aid statistical analysis.

Appendix 4.6 provides a list of SNP markers, together with their success rate, and method for selection.

4.2.6 Statistic analysis

4.2.6.1 Differences in response over time

Stundent's T-tests have already been calculated to test for significant differences in the responses at each time point throughout the FMDV peptide responses (IgG1, IgG2 and T-cell levels (section 2.3.1)) and the responses measured before and after vaccination with the BRSV vaccine (IgG1, IgG2, total IgG (section 3.3.1)). In this chapter, T-tests were also conducted to test for significance differences between each week of the IFN-γ response to the FMDV peptide (INF-γ and the T cell positive control) responses at week 0, 4 and 10.

4.2.6.2 Association Study

To conduct the Association Study (AS), all the data was normalised to give a normal distribution and constant variance, however each data set was normalised using a different method:

- The T cell response to the FMDV peptide was converted into a stimulation index, to account for the negative control. Following this, both the stimulation index of the T cell response and the antibody responses were normalised using a log10 transformation (section 2.2.5).

- The BRSV IgG responses were normalised using the logit function (section 3.2.4).

- The IFN-γ data was converted into a binary trait, with responders = 1 and non-responders =0.

For both the FMDV and the BRSV data an Area Under the Curve (AUC) measurement was taken (using the trapezoidal rule [122]) to provide a single trait that reflected the overall response of each animal.

As AS methodology does not account for relationship structure, a genetic Identity By State (IBS) matrix was constructed using all the SNP genotyped in the RoBoGen herd. The statistical analysis program, R (http://www.r-project.org/), was used to construct the IBS matrix using the GenABEL package (http://127.0.0.1:25804/library/GenABEL/html/GenABEL-package.html). To calculate the principal components the command princomp (http://127.0.0.1:25804/library/stats/html/princomp.html) was used. The principal components were all tested at each time point for significance (REML models were used). If they reached a p-value of 0.05, or they improved the Akaike's Information Criterion (AIC) [183] and were significant to a p-value of 0.1, they were retained in the REML model for that week. As a result, different combinations of the first 10 principal components were used at various time points.

The REML models used at each time point were based on the models used for chapter 2 for the FMDV peptide responses and chapter 3 for the BRSV vaccine responses. However, each model had the addition of SNP as a fixed effect and a selection of principal components 1 to 10 (to account for relationship structure of the second generation) animals. In brief the new model was:

$$Y_{ijk}= \mu +L+C+S+T+\beta_1.m+\beta_2.a+\beta_2.p +u_j+g_k+e_{ijk}$$

Where: Y_{ijk} is the observed value of the phenotypic trait; μ, population mean; L, the fixed effect of line (3 Lines); C, the fixed effect of cohort (3 Cohorts); S, the fixed effect of sex (male or female);T, the fixed effect of the SNP (AA, AB or BB); β_1, the

linear regression on the covariate of age at vaccination m (m = d469-d609); β_2, the linear regression on the covariate of weight a (a= 361-744kg); β_2, the linear regression on the covariate of a principal component p (p= -0.9-1.3); u_j, the random effect of sire; g_k, the random effect of dam g; e_{ijk}, the residual error e ~ $N(0,I\sigma^2_p)$.

The false discovery rate of the SNPs was determined using the Bonferroni correction [99] (section 1.3.3.1). For the calculation of the genome wide 5% and 1% the total number of SNP used was 'n', the number of tests (section 1.3.3.1). To calculate the chromosome wide 5% and 1% levels, only the number of SNP of each chromosome was used as 'n', thus each chromosome had a different significance threshold.

4.2.7 SNP effects and Variances

The trait values were used to estimate additive and dominance effects for each SNP. The equations used were:

Additive effect:

$$a = (AA - BB)/2$$

(However, if no BB alleles were available a=AA and dominance was not calculated)

Dominance effect:

$$d = AB - [(AA + BB)/2]$$

In both instances AA, AB and BB were the predicted trait values for each genotype class.

The additive genetic variance (V_{SNP}) for each SNP was calculated by the following equation:

$$V_{SNP} = 2pq[a + d(q - p)]^2$$

Where, p and q, were the allelic frequencies at the SNP locus.

The phenotypic variance (V_p) was obtained from:

$$V_P = V_E + V_S + V_{SNP}$$

Where, V_E is the residual variance and V_S is the sire variance. The proportion of phenotypic variance accounted for by each SNP ($\%V_{SNP}$), was calculated by the following equation:

$$\%V_{SNP} = V_{SNP}/V_P$$

4.2.8 Functional Roles of SNP

Ensembl was used to place SNPs and discover their status (synonymous/non-synonymous) and the locations of the SNP relative to gene exons. Ensembl was also used to detect homology of bovine genes with unassigned functions and to conduct BLAST searches (http://www.ensembl.org/Multi/blastview).

Significant SNPs located within TLR genes were analysed further for potential functional consequences using LRRfinder [184] and SMART [185] to locate their positions within the protein domains encoded by the TLR genes. SMART was used to predict the TIR domain of the TLR. However as this programme has been shown to miss many Leucine Rich Repeats (LRRs) within TLRs [186], LRRfinder [184] was used to predict the ectodomain structure.

4.2.9 Phylogenetic Analysis using Maximum Likelihood

An analysis of functional constraint and molecular evolution of the *TLR* genes was conducted using PAML [187].

The methods used for the calculations of the d_n/d_s (non synonymous / synonymous) and the classification of the type of selection (purifying, neutral or positive) were based on a paper by *Jann et al.*[179].

The SNP information on each animal was loaded into PAML and compared to the consensus sequence (Btau 4.0) to detect nsSNP. Ratios between synonymous and non-synonymous substitutions (ω) at Amino Acid (AA) sites were estimated. Using

maximum likelihood, the probability of each AA in a protein being one of the three possible classes (low ω (purifying selection), intermediate ω (neutral) or high ω (positively selected)) was calculated. The class with highest probability is assigned to that amino acid.

4.3 Results

4.3.1 The Traits

195 female F2, Holstein back-cross and Charolais back-cross animals were immunised with the FMDV15 peptide and 468 animals were vaccinated with the BRSV vaccine. The responses measured, across time, post immunisation to the FMDV15 peptide were: IgG1 antibody, IgG2 antibody, IFNγ and T cell responses. The T cell and IFN-γ responses to a T cell mitogen, Concanavalin A (ConA) were also measured at each time point as a positive control. The BRSV vaccinated animals also had IgG1 antibody and IgG2 antibody levels measured post immunisation across time. Thus there were a total of 8 distinct traits investigated.

The means, ranges and standard deviations of all the traits in this study can be seen in Table 4.1. Analysis of the FMDV (IgG and T cell responses) [155] and BRSV (IgG responses) datasets have already been conducted [177]. In summary, the FMDV peptide elicited both humoral and cellular immunity in all animals, although considerable variation between animals was observed. Similarly the BRSV vaccine induced variable antibody responses. Following both immunisations the IgG2 response tended to be lower than the IgG1 response

However the IFN-γ results have not been reported previously. Unlike the antibody and T cell responses [119, 155], many animals were non-responders (with IFN-γ) to the FMDV peptide at one or more time points. At weeks 4 and 10, the average response to the FMDV peptide was 388 pg/ml (standard deviation (SD) 504) and 402 pg/ml (SD 813), to Con A at the same weeks it was 418pg/ml (SD 484) and 315ng/ml (SD 323), respectively. The IFNγ responses to the positive proliferation control, Con A, were not significantly different from each other at any week, including week 0 versus week 10, as expected. However, the IFNγ response to the FMDV15 peptide was significantly different between week 0 and week 4 ($p<0.001$), week 4 and week 10 ($p < 0.05$) and also between week 0 and week 10 ($p<0.001$).

Immunisation[1]	Trait[2]	Mean[3]	Range[4]	SD[5]
FMDV peptide - IgG Responses	IgG1_week_0	1.22	0.00 - 9.31	1.36
	IgG1_week_1	1.33	0.00 - 8.86	1.402
	IgG1_week_2	69.74	0.00 - 322.86	66.16
	IgG1_week_4	166.95	0.00 - 672.03	144.39
	IgG1_week_8	291.65	0.00 - 1254.56	269.20
	IgG1_week_10	176.12	0.00 - 670.78	141.63
	IgG1_AUC	1658.47	0.08 - 6421.13	1381.31
	IgG2_week_0	0.01	0.00 - 0.35	0.05
	IgG2_week_1	0.01	0.00 - 0.49	0.0505
	IgG2_week_2	5.64	0.00 - 51.07	8.80
	IgG2_week_4	13.09	0.00 - 87.85	17.32
	IgG2_week_8	24.35	0.00 - 810.60	64.70
	IgG2_week_10	23.50	0.00 - 968.30	71.77
	IgG2_AUC	144.41	0.01 - 3622.06	302.30
FMDV peptide - Tcell Responses	SI_CA_10ug_0	13.85	0.04 - 177.04	23.37
	SI_CA_10ug_4	17.67	0.00 - 197.68	29.15
	SI_CA_10ug_8	13.35	0.02 - 95.01	16.79
	SI_CA_10ug_10	18.76	0.12 - 145.38	25.57
	SI_CA_10ug_AUC	157.43	0.00 - 1214.95	187.51
	SI_FMDV_2ug_0	1.13	0.01 - 5.32	0.58
	SI_FMDV_2ug_4	1.80	0.00 - 10.12	1.50
	SI_FMDV_2ug_8	2.57	0.37 - 28.29	3.64
	SI_FMDV_2ug_10	4.58	0.30 - 173.76	14.56
	SI_FMDV_2ug_AUC	21.83	0.00 - 289.59	27.19
FMDV peptide - IFNg Responses	SI_CA_IFN_Week_0	0.41	0.00 - 1.00	0.49
	SI_CA_IFN_Week_4	0.66	0.00 - 1.00	0.47
	SI_CA_IFN_Week_10	0.59	0.00 - 1.00	0.49
	SI_FMDV_IFN_Week_0	0.39	0.00 - 1.00	0.46
	SI_FMDV_IFN_Week_4	0.63	0.00 - 1.00	0.49
	SI_FMDV_IFN_Week_10	0.53	0.00 - 1.00	0.50
BRSV Vaccine - IgG Responses	IgG1_week_0.	13.82	0.03 - 94.03	20.40
	IgG1_week_2.	20.03	1.24 - 93.03	15.44
	IgG1_week_5.	38.96	4.03 - 110.95	19.13
	IgG1_week_7.	31.20	1.32 - 98.77	20.31
	IgG1_AUC	191.13	10.37 - 592.77	89.66
	IgG2_week_0.	2.16	0.02 - 20.14	2.57
	IgG2_week_2.	4.54	0.05 - 30.44	4.09
	IgG2_week_5.	12.55	0.15 - 86.09	10.75
	IgG2_week_7.	13.07	0.06 - 81.03	13.23
	IgG2_AUC	57.36	0.50 - 327.39	43.15

Table 4.1: Trait means, ranges and standard deviations.

1. Immunisation type and response measurement recorded.
2. Trait: each trait is shown as follows: trait type (IgG1; IgG2; SI_FMDV = T cell proliferation to the FMDV peptide; SI_CA = T cell proliferation to Concanavalin A), followed by week post immunisation.
3. Arithmetic mean of each trait. FMDV: IgG1 and IgG2 = ng/ml, T cell = Stimulation index and IFNγ = binary trait (response detected 1, no response detected 0). BRSV IgG1 and IgG2 = %relative Optical Density (OD).
4. The minimum and maximum value of each trait.
5. The Standard Deviation of the raw data**.**

4.3.2 SNP Selection

A total of 425 SNP satisfied our selection criteria from the two methods of selection (a candidate gene approach and a QTL refinement approach of previously detected QTL [155, 177], see Materials and Methods) and were used for genotyping the RoBoGen herd (Appendix 4.3). The selected SNP were genotyped onto every chromosome with the exceptions of chromosomes 1, 10, 28, which had none (Table 4.2). From the 425 SNPs, 187 satisfied all quality checks (see Materials and Methods) across the second generation (Appendix 4.3). The FMDV dataset however, consisted of only female heifers, and only 158 SNP were eligible for use, whereas 175 were eligible for use for the BRSV dataset analysis. 32 animals were removed from the FMDV dataset as having a low call rate, thus 150 animals remained for the AS analysis. Of the 468 animals in the BRSV dataset 56 animals were removed from further analysis, leaving 412 animals for the AS.

Many (238) of the selected SNP failed and particularly those across the MHC region on BTA23 where 64 SNPs out of total of 88 SNPs (73%) failed (Table 4.2). In contrast, those on BTA6 were more successful with 50 SNPs out of 87 SNPs (57%) passing quality control (Table 4.2). In general SNP failed because they were not polymorphic in the test population.

Chromosome[1]	Region Size of placed SNP[2] (No of SNP)	Region size of Candidate gene SNP[3] (No of SNP)	Total Number of SNP Selected[4]	Number of informative SNP[5] (Placed\Candidate)
2	124,356,891 (22)		22	7
3	18,470,640 (4)		4	3
4	74,028,369 (19)		19	7
5	2,778,002 (2)		2	0
6	46,463,514 (63)	35,620 (24)	87	50 (42\8)
7	82,202,084 (6)	1,093 (4)	10	8 (4\4)
8	50,098,066 (6)	9,308 (8)	14	3 (1\2)
9	30,898,749		4	2
11	11,167,186		4	3
12	9,325,153		3	1
13	5,690,060		2	2
14	22,964,520		5	3
15	51,228,249		3	2
16	20,451,130 (4)	1,982 (6)	10	1 (1\0)
17	41,556,985 (3)	1,960 (22)	25	8
18		49,723,808	24	12
19	30,057,927		12	7
20	23,935,349		25	11
21	20,428,742		5	3
22		43,595,947	11	5
23	46,056,263		88	24
24	22,912,353		11	4
25	14,110,754		12	4
26	1,590		2	1
27	14,472,101 (5)	5,944 (4)	9	5 (2\3)
29	285,885		3	1
X		106,597	9	6

Table 4.2: SNP placement.

1) Chromosomal location of SNP
2) Region size of chromosome (bp) the SNP covered (placing them under QTL from chapters two and three). Number in parenthesis highlights the number of SNP used, if SNP were placed using both methods (refining QTL and candidate gene study)
3) Region size of chromosome (bp) the SNP covered when placed within candidate genes. Number in parenthesis highlights the number of SNP used, if SNP were placed using both methods (refining QTL and candidate gene study).
4) Total number selected on the chromosome
5) Number of informative SNP genotyped on to chromosome. The parenthesis highlight the split between informative SNP selected under QTL (left) and within candidate genes (right).

4.3.3 Overall AS results

The REML models had already been optimised in previous publications [155, 177] and so each SNP was added, one by one, as a fixed effect at each time point throughout the FMDV and the BRSV datasets. This resulted in a total of 35 SNP

associations across the time courses to the immune responses of the BRSV vaccine (19 associations from 14 SNPs) and the FMDV15 peptide (16 associations from 10 SNPs) that were above the 5% chromosome wide significance level (Table 4.3). Very few SNPs were significantly associated with both FMDV and BRSV traits during the time course (Table 4.3). Only the T cell proliferation to the FMDV15 peptide and the IgG2 response to the BRSV vaccine had at least one significant SNP association at each time point throughout the study. In addition, only the early to mid (weeks 2 to week 4) IgG responses to the FMDV15 peptide had significant associations, and from all of the IFNγ measurements only two time points had significant SNP associations (Con A response at week 4 and FMDV15 response at week 10 post immunisation), Table 4.3.

The most significant SNP (rs43702384) (F statistic of 11.6; above the 1% genome wide significance level) was located on BTA6, placed in the predicted gene, glucosidase beta acid 3 (GBA3) [188]. The SNP was located in the first exon and was non-synonymous, substituting a Methionine for a Threonine (Table 4.4). It was associated with the IgG2 response to the BRSV vaccine at week 2 following vaccination and accounted for over 11% of the genetic variance. However it was not associated with any other trait. The second most significant SNP (rs42765470) (F-statistic of 9.8; above the 5% genome wide significance level) was also located on BTA6 although in a different region. It was associated with the overall T cell response (AUC measurement) to the FMDV peptide, and additionally was significantly associated with related traits for the T cell response following the boost at weeks 8 and 10. A further 2 SNPs were above the 1% chromosome wide significance level and were associated with the response to the BRSV vaccine. In addition significant SNP on this chromosome were also located under the peaks of previously detected QTL (Figure 4.1). Another SNP that was significant and place beneath a QTL was on BTA7

A SNP on BTA8 (rs8193069) was significantly associated with both the BRSV specific IgG1 (for week 5) and the IgG2 (overall) response to the vaccine. This SNP again explained a relatively large proportion of the variance, over 9% for both traits.

Additionally, a SNP on BTA 25 (rs42060100) was significantly (above 1% chromosome level) associated with the BRSV specific IgG2 response at week 5 and less strongly with the IgG1 response at week 7 (Table 4.3).

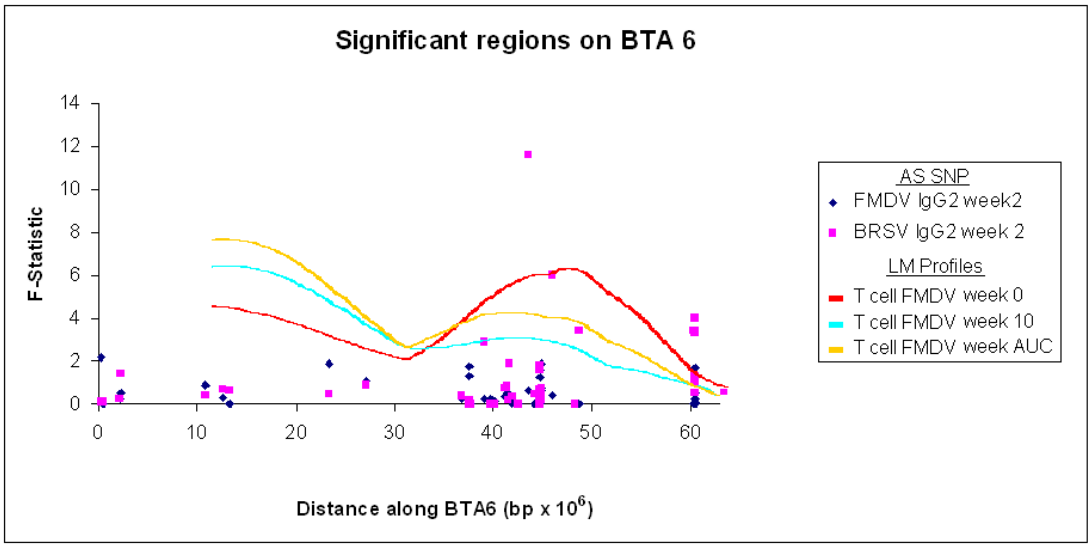


Figure 4.1: Significant regions on BTA6.
Comparison of QTL regions previously described (Chapters 2 and 3) for responses to FMDV and BRSV with significant SNP positions in this study, located on BTA6.
The previously discovered significant regions are shown using linkage mapping profiles (Chapter 2) in light blue, red and yellow. SNPs are shown as purple and dark blue points. The only significant SNP shown was associated with the BRSV IgG2 response at week 2, rs43702384, which reached an F-statistic of 11.6.

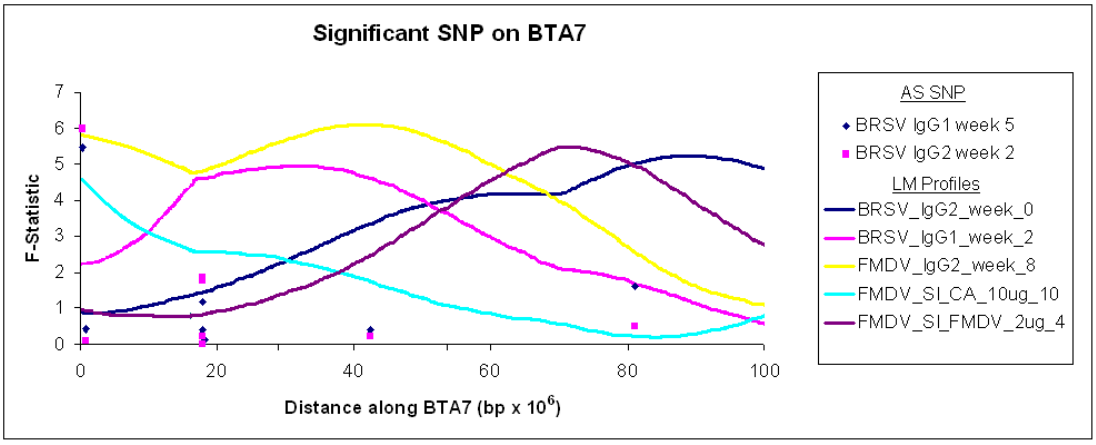


Figure 4.2: Significant regions on BTA7.
Comparison of QTL regions previously described (Chapter 3) for responses to the BRSV vaccine are compared with significant SNP positions discovered in this study, located on BTA7. The previously discovered significant regions are shown using linkage mapping profiles (Chapter 3). SNPs are shown as purple (IgG2 week 2) and dark blue points (IgG 1 week 5). Only one significant SNP was located on this chromosome, rs42371911, which was associated with the IgG1 and IgG2 responses following BRSV vaccination, with F-statistics of 6.0 and 5.5, respectively.

immunising agent[1]	Trait[2]	BTA[3]	Marker[4]	F Value[5]	snp AA ± SE[6]	snpAB ± SE[7]	Percentage of genetic variance due to SNP[8]
FMDV peptide	SI_CA_AUC	4	rs41848377	6.1	-0.28 ± 0.09	0.27 ± 0.25	2.52
	SI_FMDV_week_10	6	rs42765470	7.9	-0.06 ± 0.39	0.35 ± 0.09	0.11
	SI_FMDV_week_8	6	rs42765470	8.2	0.21 ± 0.33	0.30 ± 0.07	1.69
	SI_FMDV_AUC**	6	rs42765470	9.8	0.10 ± 0.25	0.25 ± 0.06	0.70
	SI_CA_week_4	8	rs8193069	3.8	0.90 ± 0.34	-0.08 ± 0.12	11.55
	SI_FMDV_week_4	12	rs43038502	4.8	-0.15 ± 0.08	0.08 ± 0.04	3.74
	SI_FMDV_AUC	12	rs43038502	3.9	-0.06 ± 0.08	0.10 ± 0.05	0.62
	IgG1_week_4	15	rs42205048	4.6	-0.08 ± 0.08	0.12 ± 0.07	0.77
	IgG2_week_4	19	rs41910166	5.3	-1.83 ± 0.63	0.28 ± 0.25	12.31
	IgG1_week_4	22	rs41995914	8.9	-0.20 ± 0.07		6.23
	SI_CA_IFN_Week_4	24	rs42053876	6.3	-0.18 ± 0.10	0.06 ± 0.08	3.87
	SI_FMDV_week_10	24	rs42053876	5.3	-0.22 ± 0.09	-0.27 ± 0.08	1.58
	SI_CA_week_4	26	rs42102914	3.7	-0.33 ± 0.17	-0.25 ± 0.10	3.40
	SI_CA_AUC	26	rs42102914	3.3	-0.29 ± 0.15	-0.19 ± 0.09	3.53
	SI_FMDV_IFN_Week_10	26	rs42102914	4.9	0.24 ± 0.13	0.12 ± 0.08	4.62
	IgG1_AUC	X	rs55617390	4.9	-0.30 ± 0.10	-0.02 ± 0.07	6.31
BRSV vaccine	IgG2_week_2***	6	rs43702384	11.6	-1.50 ± 0.44	-0.08 ± 0.12	11.88
	IgG1_week_5	7	rs42371911	5.5	-0.37 ± 0.24	0.19 ± 0.10	1.55
	IgG2_week_2	7	rs42371911	6.0	-0.95 ± 0.28	-0.18 ± 0.12	6.29
	IgG1_week_5*	8	rs8193069	5.9	1.34 ± 0.44	0.18 ± 0.13	9.78
	IgG2_AUC*	8	rs8193069	6.0	60.64 ± 20.12	-9.15 ± 5.66	9.74
	IgG2_week_0	12	rs43038502	3.1	-0.73 ± 0.30	-0.17 ± 0.19	2.25
	IgG2_week_0	16	rs41817317	3.2	-0.08 ± 0.31	-0.47 ± 0.19	0.02
	IgG2_week_5	19	rs41915330	5.5	-0.95 ± 0.30	-0.18 ± 0.12	6.58
	IgG2_week_7	21	rs29016829	4.8	-0.82 ± 1.29	-0.56 ± 0.18	1.82
	IgG1_week_2	22	rs41995921	5.2	-1.34 ± 0.69	0.29 ± 0.13	9.36
	IgG2_week_0	22	rs41995921	5.2	1.99 ± 1.16	0.68 ± 0.22	7.26
	IgG2_week_0	24	rs42042620	6.0	-1.37 ± 0.42	0.07 ± 0.19	6.52
	IgG1_week_7	25	rs42060100	5.6	-1.49 ± 0.52	0.16 ± 0.13	2.29
	IgG2_week_5*	25	rs42060100	6.5	-1.67 ± 0.53	0.09 ± 0.14	4.29
	IgG1_AUC	27	rs42544474	5.1	47.62 ± 16.89	0.01 ± 9.39	3.38
	IgG1_week_2	27	rs42544474	5.6	0.63 ± 0.21	0.11 ± 0.11	4.37
	IgG1_week_2	29	rs42155456	4.1	0.47 ± 0.16	0.33 ± 0.13	2.92
	IgG1_week_2	X	rs55617145	5.4	-0.53 ± 0.17	-0.19 ± 0.13	3.62
	IgG2_week_5	X	rs55617390	5.4	-0.62 ± 0.20	-0.23 ± 0.13	4.08

Table 4.3: Significant SNP associations with the immune response following immunisation with the FMDV15 peptide and the BRSV vaccine.

1. Immunisation agent: FMDV15 peptide or the BRSV vaccine.
2. Trait: as in Table 1.
3. BTA: The *Bos taurus* chromosome the SNP is located on.
4. F-value: The F-statistic for each SNP
5. Marker: SNP reference number discovered to be significantly associated with immune response.
6. Additive effect of the SNP with the standard error.
7. Dominance effect of the SNP with the standard error.
8. Percentage of the genetic variance due to the SNP.

The additive variance accounted for by each SNP varied greatly in this study (Table 4.3), with the highest being 12.31% (IgG2 response to the FMDV15 peptide at week 4) and was the only SNP to be significantly associated with the IgG2 response to the FMDV15 peptide at any time point..

4.3.4 Further Analysis of Significant SNPs

All SNPs that were above the 5% chromosome wide significance level were analysed further for their relative location to genes and to discover if they were non-synonymous (Table 4.4). The majority of significant SNPs were located within gene exons and were also non-synonymous (ns) (Table 4.4). In addition, SNP were selected (by the Perl script), in genes with unknown function, to be genotyped. Of these, some were shown to have significant associations with the immune responses measured (Table 4.4).

Two genes, with known function, which contained significant SNP within this chapter are TLR4 and TLR8 (SNP within TLR8 are shown in Figure 4.3). TLRs are one of the first defences against pathogens. Due to their direct link in eliciting an immune response, further analysis was conducted upon the SNP located within the TLR genes.

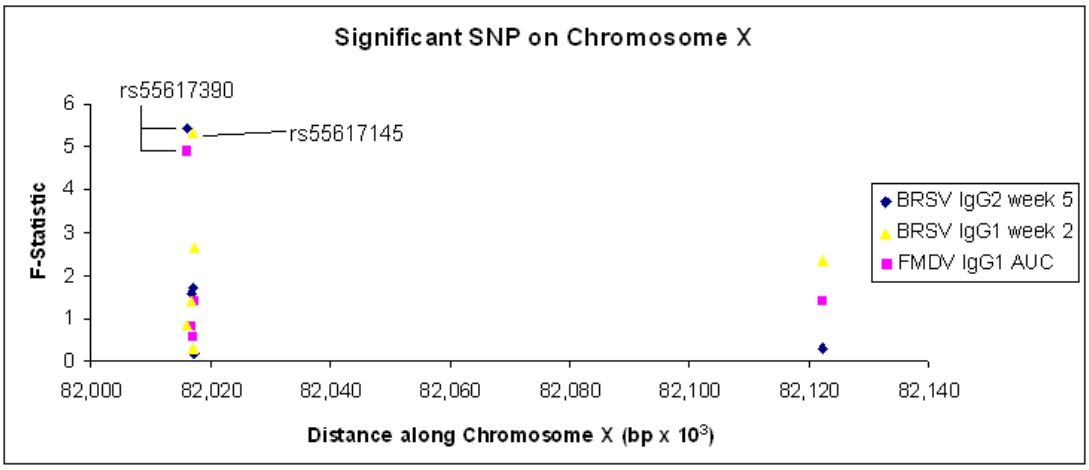


Figure 4.3 Manhattan plot of Chromosome X:
SNP on chromosome X with their F-statistic highlighting the significance to the IgG1 response to the FMDV peptide across time, pink squares (AUC). The SNP associated with the responses to the BRSV vaccine for IgG1 (yellow triangles) and IgG2 (blue diamonds) is also shown at week 2 and 5, respectively. The significant SNP are labelled and located with TLR8.

In order to explore whether the 3 nsSNPs, within TLR4 and TLR8, were likely to have functional consequences two programs were used. The precise positions of the associated nsSNP were determined using SMART [185] for the TIR domain structural analysis. LRR finder [189] was used to identify the Leucine Rich Repeats (LRRs) within their (putative) ligand binding domains. In addition, since positively selected SNPs are more likely to have functional effects [179], the selective pressure on these SNPs was also estimated.

The significant SNP, rs8193069, coded for a Threonine to Isoleucine substitution and was located at the start of the TIR domain in the bovine protein sequence of TLR4 (NP_776623) (Figure 4.4). PAML analysis determined that the SNP is likely to be under positive selection with a ω of 1.62 (p = 0.05). LRRfinder identified 23 LRR matches from the protein sequence of bovine TLR8 (NP_001029109). In addition, LRRfinder indicated that the two significant SNP located in TLR8 (rs55617145 and rs55617390) coded for amino acid exchanges within the 12th and C-terminal LRRs respectively (Figure 4.5). The former SNP (rs55617145) coded for a Histidine to Glutamine substitution which was under significant purifying selection (ω= 0.03, p=0.02) whilst the other amino acid change (Phenylalanine to Leucine substitution) coded for by SNP, rs55617390, was not under significant selection with a ω of 0.286 (p=0.15). However, a change in amino acid may change the structure, altering the TLRs affinity to bind pathogen.

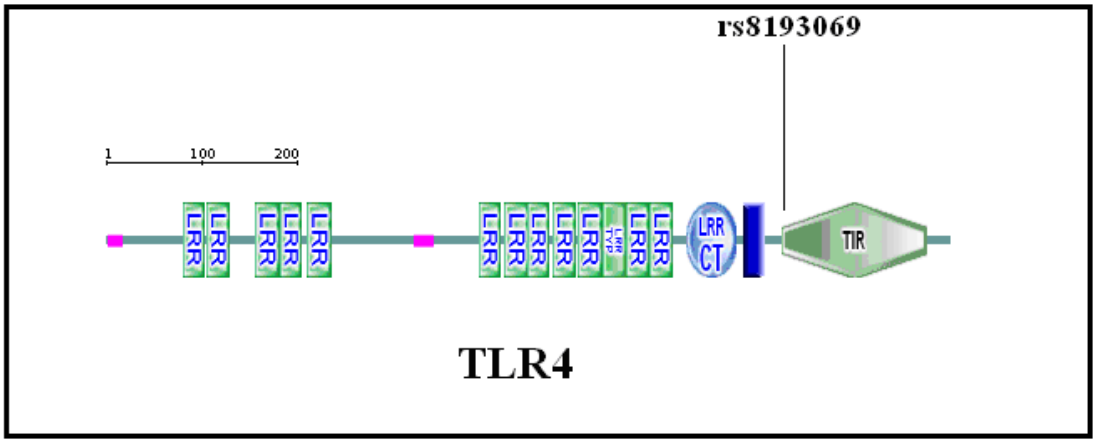


Figure 4.4: Significant SNP within the TIR domain of TLR4
Visualisation of the secondary structure of TLR4. The diagram was generated by SMART. The green rectangles are Leucine Rich Repeats. The C-Terminal Leucine Rich Repeat is depicted by a blue circle. The blue rectangle is the transmembrane region of the TLR4. The TIR domain is shown as a green diamond.

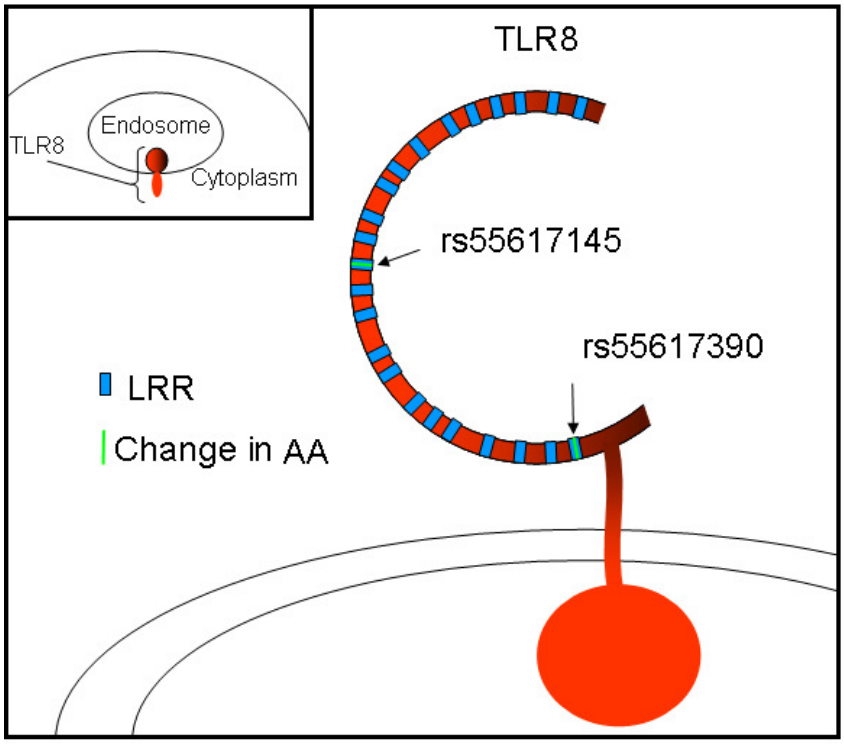


Figure 4.5: **2D Diagram of the significant SNP within the Leucine Rich Repeats of TLR8**. The visualisation of TLR8, highlighting the Leucine Rich Repeats (blue) that contain a non-synonymous SNP, which caused amino acid changes (green).

BTA[1]	SNP[2]	Location[3]	Variation State[4]	Protein Variation[5]	Gene Symbol[6]	Gene Name[7]
4	rs41848377	9,040,359	NS	G/D	ABCA13	ATP-binding cassette, sub-family A (ABC1), member 13
6	rs42765470	10,812,211	NS	S/N	ENSBTAG00000009280	similar to Armadillo Repeat Containing 1
6	rs43702384	43533566	NS	M/T	GBA3	Glucosidase, Beta, Acid 3 (cytosolic)
7	rs42371911	372053	DOWNSTREAM	NA	A6QQ78_BOVIN	Unknown. Protein 403 AA.
8	rs8193069	112,436,580	NS	T/I	TLR4	Toll-Like Receptor 4
12	rs43038502	9,310,537	UPSTREAM	NA	44bp from OLFM4	Olfactomedin 4
15	rs42205048	19,855,732	3' UTR	NA	POU2AF1 / OBF-1	Oct-Binding Factor-1
16	rs41817317	67077027	DOWNSTREAM	NA	2284bp from PTPN14	Protein Tyrosine Phosphatase, Non-receptor type 14
19	rs41910166	28,079,648	NS	P/L	Predicted: CHD3	Chromodomain Helicase DNA binding protein 3
19	rs41915330	36738471	NS	V/I	SPAG9	Sperm Associated Antigen 9
21	rs29016829	35150184	DOWNSTREAM	NA	STXBP6	Syntaxin-Binding Protein 6
22	rs41995914	11696112	NS	R/G	DLEC1	Deleted in Lung and Esophageal Cancer 1
22	rs41995921	11690241	NS	V/I	DLEC1	Deleted in Lung and Esophageal Cancer 1
24	rs42042620	30796671	DOWNSTREAM	NA	1762 bp from of CHST9	Carbohydrate (N-acetylgalactosamine 4-0) Sulfotransferase 9
24	rs42053876	38815332	UPSTREAM	NA	546 bp from MYL9	Myosin regulatory light polypeptide 9
25	rs42060100	23945463	NS	S/G	ARHGAP17	Rho GTPase activating protein 17
26	rs42102914	36858669	DOWNSTREAM	NA	3790bp from GFRA1	3790bp up sream of GDNF family receptor alpha 1
27	rs42544474	348148	DOWNSTREAM	NA	4623 from A1A4Q1	Unknown
29	rs42155456	574482	NS	P/S	similar to hCG20936	Unknown
X	rs55617145	82017087	NS	H/Q	TLR8	Toll-Like Receptor 8
X	rs55617390	82015995	NS	F/L	TLR8	Toll-Like Receptor 8

Table 4.4: SNP locations in the Bovine Genome

1. BTA: The *bos taurus* chromosome the SNP is located on
2. SNP: the SNP name
3. Location: the position of the SNP along the chromosome (in base pairs, bp)
4. Variation state of the SNP NS = Non-Synonymous within the encoded protein; Downstream = Downstream of the gene; Upstream = upstream of the gene; 3' UTR = within the 3' un-translated region.
5. Protein variation: The wild type amino acid is on the left and the substitution caused by the SNP change is on the right.
6. Gene Symbol: This is the common gene symbol for each gene.
7. Gene Name: this is the full gene name, where applicable.

4.4 Discussion

In this study we have built on our earlier results which identified QTL regions, via LM mapping, that were associated with immune related traits [155, 177]. Using a fine mapping approach with a selected SNP panel, 21 SNPs were significantly associated with the response to a 40-mer FMDV peptide [112] and a commercially available BRSV vaccine. Of the SNPs in the panel which were within the confidence intervals of the QTL discovered in the previous studies [155, 177], 18 (162 were successfully genotyped) were significant, whilst of the SNP placed in a candidate gene approach 3 (25 were successfully genotyped) were significant. Interestingly, one of the three syntenic blocks associated with cross-species QTL for disease resistance [138], was found to harbour a significant SNP (rs43702384) in the current study on BTA6, although it was not within the TLR1, 6, 10 cluster.

Previous chapters in this thesis (chapters 2 and 3) have used within-family LD to detect QTL for the immune traits used in this chapter. However, the aim of fine mapping the QTL of chapters 2 and 3 was conducted using AS methodology, which detects QTL in association with the immune traits using population wide LD. The idea of using linkage and then association methodology to fine map has been used before (see section 1.3.2.2), indeed methodology has been developed which increases the power of such a study by using linkage data to weight association p-values [190]. However, the methodology was developed for use where 100k or more markers are used, and would have very little effect on the current results, which had 183 informative SNP. AS methodology is unlikely to find a causative polymorphism (a high proportion of purported causal polymorphisms are eventually shown as false, for example: [191]) and is more likely to highlight a marker in LD with a causal polymorphism. This is reflected within the results of this chapter, although some of the SNP are indeed intriguing candidates, they are most likely in LD with a causal polymorphism, and *not* the causal polymorphism. In addition, the results of this chapter cannot be tested to investigate if the significant SNP are true associations.

Only through further validation within other populations (see section 5.3) will show if the significant SNP are in LD with a causal variants.

Perl is a programming language developed for fields such as biology as scripts can be written to solve highly computational and complex problems [192]. The script developed in the current study selected up-to-date SNPs in a species specific manner, via a connection to the Ensembl API. Since we did not have prior information about the allele frequency of the selected SNPs in the test population, SNPs were deliberately chosen in clusters with the aim that at least one SNP per cluster would be polymorphic. Thus the Perl script has the potential to select SNPs within QTL for other fine mapping projects. A limited number of SNP could be selected in the current study. Thus, the coverage of the genome was limited, compared to the coverage of the 50K SNP chip, which has a SNP placed every 50 kb (on average) (http://www.illumina.com/Documents/products/datasheets/datasheet_bovine_snp5O.pdf). The coverage of the regions of interest varied greatly in the current chapter, with some chromosomes not having any SNP placed on them, whilst some candidate genes had a SNP genotyped every ~250 bases. Further, only one SNP was selected in the current chapter that exists on the Illumina SNP chip (rs29017177); however this SNP was not successfully genotyped in the RoBoGen herd. With over 1,970,267 short variants (SNPs, indels, somatic mutations) (http://www.ensembl.org/Bos_taurus/Info/StatsTable?db=core) in the bovine genome, the probability of many SNP being in both this study and the 50K SNP chip were low.

One region expected to be significantly associated with the immune response in this study was the MHC region as previous chapters (chapters 2 and 3) and other studies had shown that QTL spanning the MHC region and polymorphisms within the *BoLA DRB3* gene accounted for some of the variation in the immune response to the FMDV peptide and BRSV vaccine [118-119, 193-194]. However no significant SNPs were discovered on chromosome 23 which may be due to the failure of most (64 out of 88 SNP on BTA23) of the SNPs across this region. The low rate of successful genotyping was caused by many of the SNP being uninformative.

Sequencing the second generation of the RoBoGen herd across the MHC region may enable the discovery of SNP, within the herd, that are polymorphic and significantly associated with the immune traits discussed in this paper.

In chapter 1 three QTL on BTA6 were associated with the T cell responses to the FMDV peptide [155]. BTA6 harbours the TLR1, 6, 10 cluster [180], however, the SNPs placed using the candidate gene methodology within this region were not significant. The Perl script selected SNP in an attempt to fine map the previously detected QTL [155, 177]. Some of the selected SNP were within genes that have no known effect on the immune response. From these SNP, two were discovered to be above the 5% genome wide significance level, both of which were located on BTA6. Both SNPs were within gene exons and were predicted to change the encoded amino acids, suggesting that they may have functional consequences. SNP rs43702384, which was above the 1% genome wide significance level, was placed in the predicted gene, glucosidase beta acid 3 (*GBA3*), and SNP rs42765470 was placed in a "known coding gene" that is similar to Armadillo repeat containing 1 (ARMC1), neither of which have been reported to have a link to immunity. The human homolog of GBA3, is suggestively involved in a nonlysosomal catabolic pathway of glycosylceramide metabolism [188] and ARMC1 is a nuclear-encoded mitochondrial protein, with only one study in the literature testing its function, finding it not significantly associated with Acquired Immune Deficiency Syndrome [195].

The Perl script also selected a SNP (rs42205048, associated with the IgG1 response 4 weeks post immunisation to the FMDV15 peptide) within the gene, B-cell-specific co-activator (OBF-1). Although the script did not select this SNP in the gene due to its 'immune relevance' (it was not within the pathways specified, see Materials and Methods) it has been shown in humans to be essential for the response of B-cells to antigens and is required for the formation of germinal centres [196]. Furthermore this transcriptional co-activator has been shown to regulate the balance between Th1 and Th2 immunity [197]. Considering the human gene shares 92% homology with the bovine gene, it is probable that bovine OBF-1 will function in a highly similar way to the human OBF-1. The SNP in this study only accounted for 0.77% of the

phenotypic variance, nonetheless its significance may point to a novel pathway that regulates immune responses and may have relevance for vaccine design. Furthermore, detailed analysis of the gene and region may reveal further SNPs which maybe closer to or in high LD with a causal polymorphism.

As well as fine mapping previously detected QTL, a candidate gene approach was used, which selected SNP within the TLR genes and their down-stream signalling partners [138, 179-180]. TLRs are strong candidates for disease resistance traits as well as targets for vaccine adjuvants [178], as they are a form of pattern recognition receptor (PRR). PRRs are essential components of the innate immune system which also help to establish an adaptive immune response [30, 139]. Many of the TLRs induce intracellular signalling to activate a type I interferon response and thus combat viral pathogens [198]. To detect pathogens, TLRs are both on the cell surface and localised within the cell.

TLR4 is an example of a cell surface TLR, that responds to gram-negative bacteria [199]. SNP within TLR4 have been associated with *Moraxella bovis,* the gram-negative bacteria which can cause infectious bovine keratoconjunctivitis [200]. Significant SNP within TLR 4 have also been associated with mastitis [201-202]. Within this study, however, we have detected that an nsSNP (rs8193069, Table 4.3) in TLR4 was significantly associated with both the IgG1 and IgG2 responses to the BRSV vaccine. Others have previously shown that TLR4 knockout mice have a decreased ability to clear RSV infections [203] and suggested that BRSV activates TLR4 [204]. Furthermore, the same nsSNP (rs8193069), has also been associated an increase in percentage of protein and fat in milk [205], and with decreased somatic cell score [206], suggesting that this SNP, could be used in dairy breeding programs for both general disease resistance and increase performance. The amino acid change caused by the SNP substitution occurs in the TIR domain of TLR4 (Figure 4.4). The TIR domain is responsible for initiating an intracellular signalling cascade to elicit an immune response [199]. The base change substitutes a Threonine, an amino acid that has a polar side chain, with an Isoleucine, which is non-polar, highly hydrophobic and larger. Further analysis revealed that this site is also under significant positive

selection, suggesting that the base change may be increasing in frequency, thus the SNP maybe very close to a causal polymorphism which affects the immune responses.

Further significant associations were also discovered in TLR8, which is an intracellular TLR that detects ssRNA [199]. TLR8 is located in a cluster on chromosome X, along with TLR7 [180], and is expressed in immune related cells such as macrophages and dendritic cells where it interacts with ssRNA to initiate an anti-viral immune response [178]. In humans however, TLR8 has been associated with susceptibility to the bacterium, *Mycobacterium tuberculosis* [207], suggesting it may play a role in defence against a wider range of pathogens. Bovine TLR8 can be activated by some TLR7 ligands and other differences in ligand interactions suggest that BoTLR8 may have subtle differences in function compared to other species [208]. Two nsSNPs (rs55617145 and rs55617390) significantly associated with the immune response in the current study are located within the TLR8 gene. Both SNPs were located within LRRs in the TLR ectodomain, a region of TLR 8 which is responsible for ligand binding [209], although the SNPs are not in LRRs previously associated with ligand binding [208]. In addition, one SNP rs55617390, was found to be significant in both the responses to the FMDV peptide and the BRSV vaccine, suggesting that this SNP association may be used by breeders to select for higher antibody levels following immunisation. Furthermore, the amino acid change caused by rs55617145 is in a site under purifying selection ($p=0.02$). The wild type allele (Histidine, coded by the A allele of the SNP) was associated with significantly higher IgG1 antibody levels than the 'C' allele of the SNP that codes for Glutamine. IgG1 may be particularly important for BRSV clearance and protection [153], and a Histidine in this position, may therefore enhance the properties of TLR8 to bind certain viral pathogens, leading to a greater adaptive response. This suggests that the 'C' allele is being reduced in frequency, which is consistent with the hypothesis that purifying selection preserves the function of most protein-coding genes [210-211]. Regions under purifying selection have also been documented in other TLRs, for example in TLR2 and TLR10 [179, 212].

4.5 Conclusion

This study has analysed the immune responses following immunisation to a FMDV15 peptide and a BRSV vaccine. Significant SNP were successfully chosen using three methodologies. Although we do not know that the significant SNP discovered in this study would influence protection, they may be functionally relevant. From the results, three significant SNPs affected the immune response to both the FMDV15 peptide and the BRSV vaccine, located within TLR4, TLR 8 and upstream from olfactomedin 4. In addition, SNP within TLR4 have also been significantly associated with different immune related traits in other herds [204, 206], suggesting this SNP as a candidate for breeding for general disease resistance. The significant SNPs discovered in this study may aid the selection of disease resistant livestock as well as suggesting new pathways that may regulate immune responses and therefore be of value for improving the efficacy of current vaccines.

Chapter 5

General Discussion

5.1 General discussion

Infectious disease of livestock continues to be a cause of substantial economic loss and has adverse welfare consequences, even in well managed agricultural systems [1]. The results and methodologies used in this thesis may provide a greater understanding of the underlying genetics of immunity which may ultimately be valuable for developing future strategies that result in breeding more disease resistant cattle and developing more efficacious vaccines.

Traditionally, breeders have selected cattle using only phenotypic merit, to produce the next generation. Modern techniques now use DNA markers, that are linked to a favourable phenotype, to aid selection [161]. The phenotypes selected for, however, such as weight gain, milk yield, growth rate, etc do not account for health traits. Thus, as these production phenotypes have been improved, immunity has not been addressed. In addition, infectious disease has continued to cause a significant loss to the cattle industry [213], in part exacerbated by modern farming methods such as feed lot herds in the United States, where many animals live in constant close proximity to each other, allowing pathogens to rapidly infect large numbers of animals. In addition, globalisation of trade is resulting in increased spread of disease, as infected cattle are transported across country borders. The discovery of genetic loci that play a role during an immune response has the potential to be exploited in the design of vaccines that enhance the host immune response to pathogens in all individuals regardless of genotype. Thus understanding the genetic controls underpinning variation in immunity is now of paramount importance.

This thesis has focused on the identification of non-MHC regions in the bovine genome determining variation in immune response and has exploited a cattle-cross resource population set up at the Roslin Institute, which was genotyped with microsatellite markers, and was extensively phenotyped for many traits. Originally the population which is a cross between a dairy breed, Holstein, and a beef breed, Charolais, was established to detect QTL for milk and meat related production traits. This approach was very successful and several QTL papers have resulted on production traits [104, 214-216], as well as coat colour [105] and temperament [106].

Further, a number of immune traits were also measured using this resource herd, including: IgG1 and IgG2 responses before and after BRSV vaccination; peripheral blood mononuclear cell proliferation in response to a mastitis causing pathogen, *Staphylococcus aureus*; IgG1, IgG2, T cell and IFN γ responses following immunisation with a FMDV peptide. Prior to this thesis, a genetic component was established for the immune response following vaccination with the BRSV vaccine [52], as well as to *Staph. aureus* [133], with both traits showing significant sire and breed effects. In addition to the microsatellite markers, the second generation cattle cross were also genotyped for the second exon of the bovine MHC (BoLA) *DRB3* gene which was associated with the immune related traits measured following immunisation with the FMDV peptide [119]. Although the FMDV traits had been measured prior to this thesis no statistical analysis of this data had been undertaken. In addition, the kinetics of the FMDV immune responses was also analysed. This PhD built on some of these earlier studies, and utilised the microsatellite markers previously genotyped as well as genotyping extra SNP to identify regions of the genome which are significantly associated with the immune responses to the FMDV peptide and the BRSV vaccine.

The RoBoGen herd was primarily established to investigate production phenotypes. Thus using the herd to study immune responses had to be considered carefully to avoid influencing the production trait measurements. In addition no resources for challenge experiments were available which further restricted the types of immunogen which could be used. Thus a commercial BRSV vaccine was used that did not need any form of extra licensing and previous experiments had established that the FMDV peptide did not cause any adverse effects [6]. In addition, both cell mediated and humoral responses were measured from blood to minimise disruption to both the collection of production phenotypes and the herd. T cell levels were measured as a correlate of the cell mediated immune response, whilst IgG1 and IgG2 levels were measured as a correlate of the humoral immune response. Although it is clear that cell mediated immunity is important in BRSV infections [149], preliminary experiments did not detect any T cell responses to the BRSV vaccine and indeed other workers have also found that considerable immunostimulation is required to

detect cell mediated responses to BRSV in blood (Glass, personal communication). Thus IgG1, which may be required for viral clearance of and protection of BRSV [153] and IgG2 which has also been correlated with protection [150], were the only phenotypes available in response to the BRSV vaccine. In contrast, the T cell response, which has been shown to play a role in protection against FMDV [115-116], was measured following FMDV immunisation along with the IgG1 and IgG2 levels which are thought to correlate with protection [113-114]. Conducting a challenge study would further the potential to discover regions of the genome which play a role in the immune response. The challenge study would select animals by their genotypes, to be predicted high and low responders to the original phenotypes measured. Following challenge with FMDV and BRSV, extra phenotypes could also be utilised to measure morbidity, such as fever, rapid breathing and appetite depression for BRSV [143], and the detection of lesions on the tongue and foot, laboured breathing and viral shedding for FMDV [2]. In addition to adding to the information available to validate and detect significant QTL/SNP, measuring extra phenotypes as well as the original phenotypes, would enable further conclusions to be drawn, for example, if the original phenotypes correlate with resistance.

In contrast to the MHC region which has been associated with many immune traits, for example: in humans [217-219], mice [220-221] and livestock [6, 43, 118] (chapter 2), the role of non-MHC regions of livestock genomes in relation to immune responsiveness and disease resistance have received less attention. However, some non-MHC regions have been associated with infectious diseases, for example: infectious bovine keratoconjunctivitis (IBK) [4, 200], mastitis [169, 205-206, 222] and Mycobacterium avium subspecies paratuberculosis [223], in addition to the regions discovered in this thesis, which were associated to FMDV peptide [155] (chapter2) and the BRSV vaccine [177] (chapter 3). One of the first non-MHC genes to be successfully associated with an immune response, in cattle, was bovine leukocyte adhesion deficiency [224]. Mutations in this gene resulted in multiple defects in leukocyte function [225]. More recently, a gene associated with the response to multiple antigen types has been TLR4. TLRs are pattern recognition receptors which detect the initial invasion of pathogens and signal alarm. The same

SNP, within TLR 4, that was significantly associated to the FMDV peptide and the BRSV vaccine [226], has also been significantly associated with mastitis [206], implying this SNP may be used to breed cattle for general disease resistance.

Investigating significant polymorphisms which affect the immune response before and after vaccination will ultimately lead to a better understanding of disease processes and the mechanisms through which pathogens evade host immunity [227]. MHC regions have been shown to significantly affect the immune response, in cattle, following vaccination [57], however, the results in this thesis, and others have shown that non MHC genes also have a significant effect on the immune response before and after vaccination [6, 8-9]. By targeting the genes causing the high/low response during vaccination, it may be possible to improve vaccine components to increase overall vaccine efficacy, regardless of an animal's genotype. For example, two SNPs, rs55617390 and rs55617145, were significantly associated with BRSV vaccination and were located within separate Leucine Rich Repeats (LRRs) of TLR8. LRRs are responsible for ligand binding [209]. As the SNPs were non-synonymous, the structure of the LRRs , at least at these sites, would likely change with the amino acid substitutions, perhaps allowing animals with the substitutions in TLR8 to bind ligands with higher affinity. Thus developing an adjuvant that will trigger TLR8 in low responding animals may improve the efficacy of the BRSV and other viral vaccines.

The published studies of non-immune traits within the RoBoGen herd, used linkage analysis (LM) methodology to detect QTL, as the herd was produced as a typical F2 and backcross design, and genotyped with microsatellite markers. These studies were based on the assumption that genes underpinning dairy and beef traits would be fixed in the two founding breeds (Holstein and Charolais respectively) and indeed significant QTL relating these traits were discovered. However, nothing was known about the differences in the immune traits in the founder breeds. It is unlikely that the genes controlling the immune traits were fixed in the founder breeds thus making it more difficult to detect QTL in the RoBoGen herd. Nonetheless, significant QTL were detected as described in this thesis. Furthermore, no single breed was

responsible for a significant majority of QTL detected. Implying there is little difference between the immune systems of the Holstein and Charolais breeds.

The publications using the RoBoGen herd show the versatility it possesed, as studies investigated multiple production traits [104-106, 228] and immune related traits [52, 118-119, 133, 155, 177, 226, 229], with a focus on both quantitative genetics, QTL discovery and the discovery of significant effects during vaccination and infection. However, the herd was established over 10 years ago and methodology and marker technology has progressed along way since (see chapter 1). Modern resource herds tend to be much larger and have many more markers genotyped across their genomes, greatly increasing the power to detect QTL. In addition, money can be saved, using modern herds, by genotyping across one generation as AS approaches do not need detailed pedigreed information across generations.

This thesis has concentrated on analysing the genetic effects of two sets of immune-related data. At the start of the AS study, the data analyses took a relatively long time to complete. However as I developed my scripts, the time taken to complete computations was drastically reduced. With around 500 animals, several time points, and two immunogens, the trait data sets would take around 2 hours to calculate the significance of all the SNPs using the AS methodology discussed in chapter 4. In addition, empirically calculating significance thresholds for SNP [100], took around 12 hours per immunogen. However as I developed my scripts to use vectors for repetitive calculations rather than loop structures [192, 230-231], and use parallel computing [232], at the Roslin site, the computational time was reduced to around a quarter of the original time. A further issue was computational capacity which became a limiting factor in terms of how much data could be generated. For example, empirically calculating significant thresholds of SNP via permutation of the phenotypes and genotypes would create up to 2 GB of data per time point, per trait. Adjusting my scripted programs to run more efficiently, reduced the amount data that was stored, thus, if a result was stored, yet only used once at the beginning of a calculation, deleting it after it was used saved space.

5.2 Future perspectives

There are many ways to continue exploiting the genomic data of the RoBoGen herd to further detect loci/markers that are associated with the immune response following immunisation. Discussed below are a few possibilities in which the RoBoGen herd could continue to be investigated. In addition, there are a few ideas which would require further resources, but are still within the remit of the thesis hypothesis.

As the RoBoGen herd no longer exists, collecting further phenotypes is not possible. However, as many phenotypes were measured including performance traits such as carcass weights and growth rates, it would be possible to address the controversy surrounding the relationship between production traits and immune related traits [233]. In addition, with the genomic data already collected from the RoBoGen herd, investigating the association of markers with both production and immune related phenotypes may highlight the potential of breeding for both immunity and production. Results from chapter 4 have shown that a marker in TLR4 (rs8193069), is associated with both the FMDV peptide and the BRSV vaccine. The same marker has also been associated reduced with somatic cell score [206] and increased percentage of protein and fat in milk [205]. This suggests that for the SNP, within TLR4 at least, it would be possible to breed cattle for both production and health traits.

Although no further phenotyping is possible, extensive DNA banks are still available for further analysis. Thus further genotyping of the RoBoGen herd to discover novel SNP associations with the immune-related phenotypes would be possible. However, the herd had few founders and its size is relatively small, compared to modern herds. Thus, extensive genotyping would likely lead to highlighting haplotype blocks, and not novel SNP associations. This would likely occur if the herd was genotyped with a 50,000 SNP chip (personal communication, Dr. De Koning). However, as only a small amount of the phenotypic variation has been accounted for within chapter 4, relative to the peak heritability's (BRSV, 0.36 [52], and FMDV, 0.44), further genotyping the RoBoGen herd should be successful. Extra SNPs would be best placed within regions of the genome which have already been associated with an

immune response in other herds or species, as shown by Jann *et al* [138]. In addition, also placing SNP into candidate genes which are involved in mediating immune responses elicited by FMDV and BRSV infections, such as Interferon α [234], double stranded RNA-dependent protein kinase (PKR) [235] and αvβ6 integrin receptor [134] will also increase the potential of discovering novel SNP associations.

Further genotyping would increase the power of future genetic studies, increasing the probability of detecting significant SNP associations, and possibly enabling further forms of genetic analysis, for example, with extra SNPs, epistatic effects (section 5.2.1.1) may be detectable within the herd.

5.2.1 A wider search

Using immune-related phenotypes already collected from the RoBoGen herd to detect different forms of gene-environment interaction may highlight novel gene interactions during an immune response following vaccination. Below, different approaches that could be used on the RoBoGen resource are outlined. In addition, further genotyping would benefit the following techniques.

5.2.1.1 Epistasis

Epistasis is the interaction of two genes. It refers to the changes in phenotypic effects of one gene caused by that of another gene [69]. As the immune response is highly complex, interactions between regions of the genome involved in eliciting an immune response is highly likely, with the MHC region in cattle being a good candidate, as epistatic effects have been discovered in the human MHC [236]. Investigating interactions between SNP could implicate SNP that individually are not significant, yet when combined together explain a disproportionately large amount of the phenotypic variance. However, as many calculations are made to test for an interaction between every loci, detecting epistasis can be extremely computationally exhaustive, especially a on genome wide scale [237]. However, with the current level on markers genotyped in the RoBoGen herd, computation should not be an issue.

Discovering significant interactions may highlight novel pathways and genes involved in the immune response following immunisation.

Time was the limiting factor which meant that epistasis was not investigated during this thesis. To begin analysis no extra data would be required. Detecting epistasis using the RoBoGen herd, which is a relatively small herd, with few founders would be improbable due to the lack of markers and the expected existence of large haplotype blocks (as previously mentioned). However, detecting epistasis in highly polymorphic regions such as the MHC region may be possible.

5.2.1.2 Time modelling

As the immune phenotypes in this study were measured across time, further modelling is possible which may generate new phenotypes for more precise QTL detection. Throughout chapters 2 to 4, some time modelling, was completed, such as the "Area under the Curve" calculations [122]. These gave one concise measurement for each animal which summarised that animals' response across time. This was successful and did discover QTL (see chapters 2-4) that could be valuable to animal breeders and vaccinologists as the underlying regions of these QTL have a sustained effect throughout an immune response. However the AUC method is relatively crude and does not indicate any fluctuations in response an animal may have had. A different method of modelling time, which tabulated each week, was employed in Chapter 2. This enabled a comparison between traits in terms of their responses across time. Thus by including week into the REML model it was possible to test if, for example, breed had an effect on the IgG1 levels significantly increasing or decreasing at specific weeks. However few significant results were obtained and these additional steps were only implemented in Chapter 2.

The two forms of time modelling mentioned above were successful to varying degrees, suggesting more precise forms of time modelling may discover further significant and novel QTL/SNP associated with the immune response. Methods such as random regression and Gompertz curve regression may be applicable to this dataset. These forms of regression have been used to map many time dependant traits

in livestock (for example, [238-240]), and the same form of sigmoid curve is seen in the immune response following immunisation. As a result a sigmoid curve would probably 'fit' the data well. Thus if the curve was mathematically integrated to obtain the area beneath the curve, a better representation of the immune response over time would be available. Also both suggested curves would allow extra phenotypes to be calculated, such as time of initial immune response, time at the maximum immune response and the point at which the immune response is being elicited at its fastest rate. Thus SNP associated with the maximum rate of responses, for example, may enable breeders to select for faster reacting immune responses. In addition, extra phenotypes would have the advantage of further explaining the variance in terms of QTL.

5.2.2 Additional Phenotypes

As previously described in Chapter 1, many phenotypes were measured in the RoBoGen herd, including further immune related phenotypes (antibody responses to a Bovine parainfluenza-3 virus and bovine herpes virus-1 vaccine and peripheral blood mononuclear cell proliferation to *Staphylococcus aureus*). As scripts have now been written, to obtain, analyse and ensure the quality and significance of QTL and SNP, from the RoBoGen source (outlined in Chapter 4), the amount of time spent analysing the extra phenotypes would be minimal. From this analysis further SNP/QTL may be discovered that are significantly associated with a number of immune responses, further highlighting regions for breeding and vaccine development.

5.2.3 Molecular studies

The statistics obtained during this thesis show that SNP and QTL are significantly associated to the immune responses elicited to a FMDV15 peptide and a BRSV vaccine. Breeders may be able to use these SNP, post validation (see validation section 5.3). This study does not, however, show how the interactions between genes and thus translated proteins change the phenotype to be more desirable to breeders. From an academic and industry standpoint, further study of the QTL and significant

SNP in this thesis should be continued to discover, unequivocally, what occurs during immunisation. Once key pathways and genes are highlighted, they can be targeted to develop more efficacious vaccines and adjuvants.

There are many different forms of molecular study that could be used to provide biological context to the discoveries within this thesis. Outlined below are a few methods that could be used to further investigate the major findings of this Ph.D.

- Transfecting the significant SNP variants of bovine TLR4 and TLR8 and comparing the variants' ability to bind pathogen (using a ligand binding assay and reporter assay) against that of the wild type would show if any functional change had occurred. This experiment would show that the TLR SNP variant has the potential to cause a change in how an immune response is elicited.

- If a significant change in the ability to bind is detected, it would be important to test whether the variants had effects in the target species. Thus APCs (such as dendritic cells and macrophages) from animals which possess the SNP variants could be incubated with appropriate ligands and functional readouts performed. For example a microarray containing the downstream genes of the TLR pathway would be able to detect if levels of gene expression were higher or lower, when binding to different pathogens, in the APCs containing the SNP variants. This experiment would determine whether the SNPs in TLRs are likely to influence relevant immune outcomes in the target species.

5.3 Validation and Commercial Applications

Cattle breeders use genomic data in two main ways. The first, involves discovering a marker in LD with a causal polymorphism. Exploitation of such markers is termed Marker Assisted Selection (MAS) and is used to breed animals for the best possible phenotypes [101] (further detail in section 1.3.4).

Before a novel SNP association or QTL can be used in a commercial capacity its association must be validated, to confirm the SNP exists in other populations. Thus once a SNP/QTL of significance is associated with a beneficial trait in a single population, the next step is to validate that SNP by checking that it has the same effect within another population, using the same phenotypes. If such SNPs are significantly associated with the desired phenotype in other herds then they could be utilised by breeders. However, before the SNP will be used on a commercial basis (for example, in a SNP chip) it must also be checked against other selection criteria. If for example, the SNP has a positive effect on antibody levels following vaccination, but it also has a detrimental effect on the growth rate of the animal, then the economic needs of cattle breeders will be taken into consideration [101].

The second form of selection is termed Genomic Selection as it uses all the markers available. GS uses markers (usually SNP) to calculate genomic breeding values (GEBV) [161], which are the sum of all the SNP effects obtained regressing the phenotype of interest on all SNPs, simultaneously [241]. Usually either Bayesian or genomic BLUP techniques are required to achieve this, as generally many more SNPs are fitted than there are animals. Once GEBVs have been calculated, only marker information is need for the next generation, thus cattle can be selected before they are sexually mature [161]. This can greatly speed up the selection and production of cattle.

The markers which have been significantly associated with immune traits in this thesis are ideal candidates to be included in a SNP panel for MAS. Although both MAS and GS are currently used in livestock to breed for growth rate, milk yield, meat quality and other economically important traits, neither have been developed commercially to improve immune related traits. As immune related traits are now becoming more important to farms and governments, these forms of selection may be an ideal way to increase disease tolerance and resistance.

5.4 Conclusion

This investigation has discovered regions of the bovine genome that are significantly associated with the immune response before and after immunisation. Initially, broad regions of the bovine genome were discovered, which were associated with immune responses following immunisation with a FMDV15 peptide (Chapter 2) and a BRSV vaccine (Chapter 3). The knowledge gained from these chapters was used to refine the QTL by genotyping SNPs within the QTL confidence intervals and by genotyping SNPs within candidate genes. This led to significant SNP associations with the immune responses elicited to either the BRSV vaccine or the FMDV15 peptide. In addition, SNP were also discovered to be significantly associated to both immunogens, possibly highlighting common genes/pathways used in the defence of multiple pathogens.

Although this thesis has discovered multiple SNP/QTL associated to the immune response before and after vaccination with the BRSV vaccine and following immunisation with the FMDV peptide, further work is warranted to fully understand the DNA variants which cause the animal to animal variance detected in an immune response. However, the results presented in this thesis have the potential to contribute, at least in part, to the design of more efficacious vaccines and towards the SNP panels that animal breeders may use to select animals with higher immune responses, following immunisation.

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Appendix

Appendix 2.1

BTA	Markers and distances between (cM Kosambi)	BTA	Markers and distances between (cM Kosambi)
BTA 1	BM6438 3.3 TGLA49 33.7 BMS4017 14.0 TGLA57 19.6 INRA128 19.4 BM864 29.2 CSSM19 23.1 BMS4044	BTA 16	BM121 12.0 TGLA53 15.8 ETH11 28.0 BM719 7.6 BM1706 12.1 HUJ625 12.1 DIK4011
BTA 2	TGLA431 32.6 CSSM42 22.2 BM4440 26.3 TGLA226 33.4 BM2113 9.8 IDVGA2	BTA 17	URB48 28.6 BMS1373 13.2 TGLA231 8.9 IDVGA40 25.8 INRA25 12.5 BM1233
BTA 3	ILSTS96 24.1 TGLA263 13.2 INRA123 0.1 INRA130 15.0 IOBT250 11.1 HUJ1177 12.4 IDVGA35 22.6 IDVGA27	BTA 18	IDVGA31 17.7 ABS13 13.2 INRA121 12.4 HAUT14 25.8 DIK67
BTA 4	BMS1788 62.3 MAF50 30.0 DIK26 3.8 IDVGA51 16.4 RM88 15.8 MGTG4B	BTA 19	HEL10 27.6 BMS2142 25.8 CSSM65 18.8 ETH3
BTA 5	BM6026 18.4 RM103 10.2 BM321 7.8 DIK4782 15.3 BR2936 4.7 ETH10 11.3 IGF1 10.1 DIK5104 19.3 ILSTS034 19.2 ETH152	BTA 20	BM3517 31.4 TGLA126 14.7 DIK15 20.6 BM5004
BTA 6	DIK5076 32.6 BM1329 13.3 DIK1054 10.4 DIK82 9.5 DIK2320 15.3 CSN3 9.4 BP7 16.1 DIK1180 2.9	BTA 21	HEL5 69.4 TGLA337 15.4 IDVGA39
BTA 7	BP41 9.5 RM6 44.4 BM1853 30.2 ILSTS6 9.4 INRA53	BTA 22	DIK1161 19.5 BMS742 9.0 BM3406 13.3 BM3628 7.0 DIK2443 11.2 HAUT24 10.5 UWCA49 13.1 DIK5307
BTA 8	IDVGA11 19.7 DIK106 37.3 HUJ174 23.1 HEL9 17.3 DIK74 21.6 CSSM47	BTA 23	IOBT528 80.3 BMS2269 7.1 BM1905
BTA 9	ETH225 16.6 BM2504 23.9 UWCA9 28.3 MM12E6 9.1 INRA84	BTA 24	TGLA351 11.1 CSSM23 17.1 ILSTS101 21.5 INRA90
BTA 10	DIK5169 26.9 BMS528 26.8 TGLA378 12.7 BM888 21.4 CSRM60 8.3 MNB-78 17.5 TGLA272 8.5 CSSM46	BTA 25	BM4005 17.5 BM737 17.1 INRA222
BTA 11	BM716 17.0 INRA177 18.1 ILSTS100 28.5 IDVGA3 11.0 HUJV174 15.0 BMS607	BTA 26	ABS12 13.5 HEL11 15.1 RM26 10.4 IOBT730
BTA 12	BMS410 26.4 BMS2057 17.4 RM162 13.4 BM6404 11.5 DIK4028 13.5 INRA5 12.4 URB054 15.3 INRA209	BTA 27	BM3507 15.8 RM209 48.8 BM203
BTA 13	HUJ616 10.9 DIK54 22.3 ABS10 18.0 DIK93	BTA 28	BP23 19.3 IDVGA43 7.6 BMS2658 7.5 IDVGA8
BTA 14	CSSM66 34.2 RM11 23.8 PZ271 10.3 BM4513 5.4 BM2934	BTA 29	TGLA86 21 RM44 10.4 MNB-166 8.3 DIK94 27.8 MNB-101
BTA 15	BR3510 20.6 JAB1 11.7 BMS2684 5.7 IDVGA10 21.9 DIK2634 20.7 BMS429		

Linkage Map
Marker distances (cM Kosambi) are shown for the sex-average maps built for the Charolais × Holstein population used in this study.

Appendix 2.2

Chromosome	Trait	cM	F	Flanking Markers	95% C.I. of QTL Position	a	d	a/SD	d/SD
2	IgG1_week_10	38 cM	5.6	CSSM42 - BM4440	20.0 - 124.0 cM	-0.211**	0.2602**	-0.19	0.16
3	IgG2_week_2	58 cM	5.93	IOBT250 - HUJ177	0.0 - 97.5 cM	0.6625**	0.3965	0.23	0.10
4	IgG1_week_1	31 cM	5.35	BMS1788 - MAF50	0.0 - 112.5 cM	-0.6298**	0.3386	-0.22	0.07
4	IgG1_week_8	20 cM	5.17	BMS1788 - MAF51	0.0 - 128.0 cM	0.1226	0.1702**	0.12	-0.20
4	IgG1_week_10	27 cM	4.92	BMS1788 - MAF52	0.0 - 112.0 cM	0.2256	-0.4727*	0.14	-0.18
4	IgG1_AUC	28 cM	6.37*	BMS1788 - MAF53	0.0 - 113.0 cM	0.1093	-0.4275*	0.10	-0.24
4	SI_FMDV_2ug_0	67 cM	4.79	MAF50 - DIK26	0.0 - 128.0 cM	-0.0034	-0.1409**	-0.01	-0.22
5	SI_CA_10ug_0	68 cM	8.10*	IGF1 - DIK5104	11.0 - 116.0 cM	-0.1326*	0.2361**	-0.17	0.22
5	SI_CA_10ug_0	100 cM	5.76	ILSTS034 - ETH152	11.0 - 116.0 cM	0.2188**	-0.1377	0.20	-0.11
5	SI_CA_10ug_10	76 cM	5.26	IGF1 - DIK5104	0.0 - 116.0 cM	-0.0535	0.2264**	-0.07	0.22
5	SI_CA_10ug_AUC	69 cM	5.89	IGF1 - DIK5105	0.0 - 97.0 cM	-0.0604	0.1745**	-0.10	0.21
6	SI_CA_10ug_0	30 cM	8.69**	DIK5076 - BM1329	16.0 - 90.5 cM	0.2569***	0.0755	0.29	0.06
6	SI_FMDV_2ug_8	59 cM	6.49	DIK82 - DIK2320	0.0 - 133.0 cM	0.1393**	-0.0821	0.26	-0.10
6	SI_FMDV_2ug_8	133 cM	7.35*	BMS739 - BM2320	0.0 - 133.0 cM	0.0372	-0.1994***	0.05	-0.25
6	SI_FMDV_2ug_10	3 cM	6.61	DIK5076 - BM1329	0.0 - 133.0 cM	0.1193**	-0.1469*	0.19	-0.17
6	SI_FMDV_2ug_AUC	2 cM	7.91*	DIK5076 - BM1329	0.0 - 133.0 cM	0.0956***	-0.0676	0.03	-0.15
6	SI_FMDV_2ug_AUC	133 cM	6.21	BMS739 - BM2320	0.0 - 133.0 cM	0.028	-0.133**	0.06	-0.26
7	IgG2_week_8	0 cM	6.24	BP41 - RM6	0.0 - 92.0 cM	-0.8466***	0.1305	-0.25	0.03
7	SI_CA_10ug_10	0 cM	4.89	BP41 - RM6	0.0 - 92.0 cM	0.0548	0.2805**	0.06	0.22
7	SI_FMDV_2ug_4	54 cM	5.45	BM1853 - ILSTS6	0.5 - 90.0 cM	-0.1089**	-0.0935	-0.21	-0.13
9	IgG1_week_0	40 cM	5.29	BM2504 - UWCA9	4.5 - 77.0 cM	-0.2948*	0.3467	-0.15	0.14
9	SI_FMDV_2ug_10	63 cM	4.92	UWCA9 - MM12E6	1.5 - 77.0 cM	0.0483	-0.2287**	0.06	-0.21
11	IgG1_week_0	0 cM	5.26	BM716 - INRA177	0.0 - 83.0 cM	0.2478	-0.6077*	0.11	-0.18
12	IgG2_week_8	17 cM	5.44	BMS410 - BMS2057	0.0 - 109.0 cM	-0.0329	1.2374***	-0.01	0.24
13	IgG1_week_1	0 cM	5.08	HUJ616 - DIK54	0.0 - 48.0 cM	0.0409	-0.5416**	0.02	-0.23
14	IgG2_week_0	2 cM	4.85	CSSM66 - RM11	0.0 - 73.0 cM	0.1229	-0.2805**	0.12	-0.21
15	IgG2_week_8	13 cM	5.16	BR3510 - JAB1	0.0 - 80.0 cM	-0.0773	1.0312**	-0.02	0.23
15	IgG2_AUC	27 cM	5.39	JAB1 - BMS2684	0.0 - 60.0 cM	-0.0844	0.6731**	-0.04	0.23
16	IgG2_week_2	76 cM	6.51	HUJ625 - DIK4011	14.0 - 87.0 cM	-0.7107***	-0.1614	-0.26	-0.04
16	SI_FMDV_2ug_AUC	64 cM	4.42	BM1706 - HUJ625	0.0 - 87.0 cM	-0.0626*	-0.0715*	-0.17	-0.15
18	SI_CA_10ug_10	22 cM	7.02*	ABS13 - INRA121	9.0 - 69.0 cM	-0.2026***	0.0957	-0.26	0.08
18	IgG2_week_1	57 cM	5.23	HAUT14 - DIK67	0.0 - 65.0 cM	-0.1001	0.3403**	-0.09	0.20
19	IgG2_week_2	28 cM	4.93	BMS2142 - CSSM65	0.0 - 72.0 cM	-0.5022*	-0.6155*	-0.17	-0.16
19	IgG2_week_4	34 cM	5.64	BMS2142 - CSSM65	11.0 - 72.0 cM	-0.7292***	-0.316	-0.23	-0.07
19	IgG2_week_8	50 cM	5.28	BMS2142 - CSSM65	0.0 - 72.0 cM	-0.8123**	-0.245	-0.20	-0.03
19	SI_CA_10ug_8	32 cM	8.57*	BMS2142 - CSSM65	16.0 - 44.0 cM	0.2462***	-0.0652	0.28	-0.06
19	SI_CA_10ug_10	32 cM	4.54	BMS2142 - CSSM65	0.0 - 72.0 cM	0.152*	-0.1262	0.18	-0.11
19	SI_CA_10ug_AUC	28 cM	4.42	BMS2142 - CSSM65	0.0 - 72.0 cM	0.1337**	-0.0098	0.21	-0.01
20	IgG1_week_2	28 cM	4.74	BM3517 - TGLA126	5.5 - 41.0 cM	-0.0277	0.4757**	-0.02	0.22
20	IgG1_week_4	36 cM	7.86*	TGLA126 - DIK15	11.0 - 46.0 cM	0.2017**	0.3526**	0.18	0.21
20	IgG1_week_8	9 cM	6.49*	BM3517 - TGLA126	0.0 - 32.0 cM	0.1919	0.6353**	0.10	0.22
20	IgG1_week_10	23 cM	5.37	BM3517 - TGLA126	11.0 - 46.0 cM	0.092	0.3801**	0.08	0.21
20	IgG1_AUC	20 cM	10.07***	BM3517 - TGLA126	12.0 - 41.0 cM	0.1198	0.3878***	0.13	0.28
20	IgG2_week_4	32 cM	7.48	TGLA126 - DIK15	20.0 - 66.0 cM	0.5221**	0.6525*	0.20	0.18
20	IgG2_week_8	25 cM	8.46	BM3517 - TGLA126	5.0 - 42.0 cM	0.7569**	0.8703*	0.23	0.17
20	IgG2_week_10	31 cM	6.23	BM3517 - TGLA126	0.0 - 56.0 cM	0.3282	0.8229**	0.12	0.21
20	IgG2_AUC	31 cM	8.5*	BM3517 - TGLA126	22.0 - 43.0 cM	0.3963**	0.674**	0.19	0.22
20	SI_FMDV_2ug_0	61 cM	7.95*	DIK15 - BM5004	32.0 - 66.0 cM	0.0515	-0.179***	0.11	-0.26
21	IgG1_week_2	69 cM	4.96	TGLA337 - IDVGA39	0.0 - 84.0 cM	-0.2211*	0.3767*	-0.15	0.18
21	SI_CA_10ug_AUC	84 cM	4.1	TGLA337 - IDVGA39	0.0 - 84.0 cM	-0.144**	-0.0089	-0.20	-0.01
23	IgG1_week_0	78 cM	5.11	IOBT528 - BMS2269	0.0 - 87.0 cM	-0.1906	-0.5408**	-0.10	-0.21
23	IgG1_week_1	65 cM	5.83	IOBT528 - BMS2269	5.5 - 87.0 cM	-0.1315	-0.8739***	-0.05	-0.24
23	IgG1_week_2	39 cM	4.16	IOBT528 - BMS2269	0.0 - 87.0 cM	0.3748	-0.5323	0.14	-0.12
23	IgG1_week_10	18 cM	5.25	IOBT528 - BMS2269	0.0 - 75.5 cM	0.3744**	0.0313	0.23	0.01
23	IgG1_AUC	18 cM	5.34	IOBT528 - BMS2269	0.0 - 85.0 cM	0.2942**	0.0637	0.23	0.03
23	IgG2_week_4	0 cM	5.45	IOBT528 - BMS2269	0.0 - 87.0 cM	0.6304**	-0.1846	0.22	-0.05
23	IgG2_week_8	5 cM	5.85	IOBT528 - BMS2269	0.0 - 87.0 cM	0.8834***	-0.1815	0.23	-0.03
23	IgG2_week_10	33 cM	4.08	IOBT528 - BMS2269	0.0 - 87.0 cM	1.0221**	0.7745	0.20	0.09
23	IgG2_AUC	6 cM	6.61*	IOBT528 - BMS2269	0.0 - 77.0 cM	0.6585***	-0.1774	0.25	-0.04
24	IgG1_week_8	35 cM	7.83**	ILSTS101 - INRA90	0.0 - 47.0 cM	0.5782***	0.3195	0.27	0.11
24	IgG1_week_10	33 cM	4.18	ILSTS101 - INRA90	0.0 - 49.0 cM	0.2724**	0.0626	0.21	0.04
24	IgG1_AUC	33 cM	6.23	ILSTS101 - INRA90	0.0 - 49.0 cM	0.2477***	0.1242	0.24	0.09
24	IgG2_week_10	21 cM	5.09	CSSM23 - ILSTS101	0.0 - 49.0 cM	0.6922**	0.6088	0.20	0.13
24	SI_CA_10ug_4	34 cM	5.46	ILSTS101 - INRA90	6.0 - 48.0 cM	-0.0479	0.2814**	-0.05	0.22
25	IgG1_week_0	0 cM	4.48	BM4005 - BM737	0.0 - 34.0 cM	0.3358*	0.2765	0.18	0.12
25	IgG1_week_1	0 cM	4.88	BM4005 - BM737	0.0 - 34.0 cM	0.2916*	0.3511*	0.17	0.15
25	IgG1_week_2	33 cM	4.42	BM737 - INRA222	0.0 - 34.0 cM	0.0512	-0.3514**	0.02	-0.19
25	IgG2_week_1	15 cM	4.48	BM4005 - BM737	0.0 - 24.0 cM	0.1819**	-0.1059	0.19	-0.08
25	IgG2_week_2	34 cM	4.65	BM737 - INRA222	0.0 - 34.0 cM	-0.1449	-0.726**	-0.06	-0.21
25	IgG2_week_4	34 cM	4.85	BM737 - INRA222	0.0 - 34.0 cM	-0.1395	-0.6993**	-0.06	-0.22
25	IgG2_AUC	34 cM	5.28	BM737 - INRA222	0.0 - 34.0 cM	-0.0364	-0.6211**	-0.02	-0.23
25	SI_CA_10ug_10	17 cM	5.79	BM4005 - BM737	0.0 - 34.0 cM	-0.1101*	0.1886**	-0.15	0.19
26	IgG1_week_1	21 cM	4.9	HEL11 - RM26	0.0 - 39.0 cM	-0.3017*	0.3489	-0.14	0.17
27	IgG2_week_4	7 cM	4.61	BM3507 - RM209	0.0 - 64.0 cM	-0.5669**	-0.3348	-0.21	-0.08
29	SI_CA_10ug_0	0 cM	5.34	BP23 - IDVGA43	0.0 - 67.0 cM	0.1542**	-0.1116	0.20	-0.11
29	SI_FMDV_2ug_8	7 cM	4.9	BP23 - IDVGA43	0.0 - 64.0 cM	-0.0983*	0.1157	-0.17	0.14
29	SI_FMDV_2ug_10	0 cM	4.38	BP23 - IDVGA43	0.0 - 59.5 cM	-0.107**	0.0825	-0.19	0.07

Supplementary table showing extra detail of each QTL:

1. Chromosome: the chromosome number of the QTL. Underlined if 2 QTL model.
2. Trait: each trait is shown as follows: trait type (IgG1; IgG2; SI_FMDV = T cell proliferation to the FMDV peptide; SI_CA = T cell proliferation to Concanavalin A), followed by week post immunisation.
3. cM: the position the QTL is on the chromosome, in centiMorgans.
4. F: the F-statistic for each QTL. Significance level: all are at least 5% chromosome wide, * =p<1% chromosome wide, **=p<5% genome wide and ***=p<1% genome wide.
5. Flanking markers of each QTL peak.
6. The 95% confidence intervals of each QTL.
7. “a” and “d” are the additive and dominance effect, respectively, of each QTL, * =p<5%, **=p<1% and ***=p<0.01%.
8. “a/SD” and “d/SD” are the standard deviation units for the additive and dominance effects, respectively.

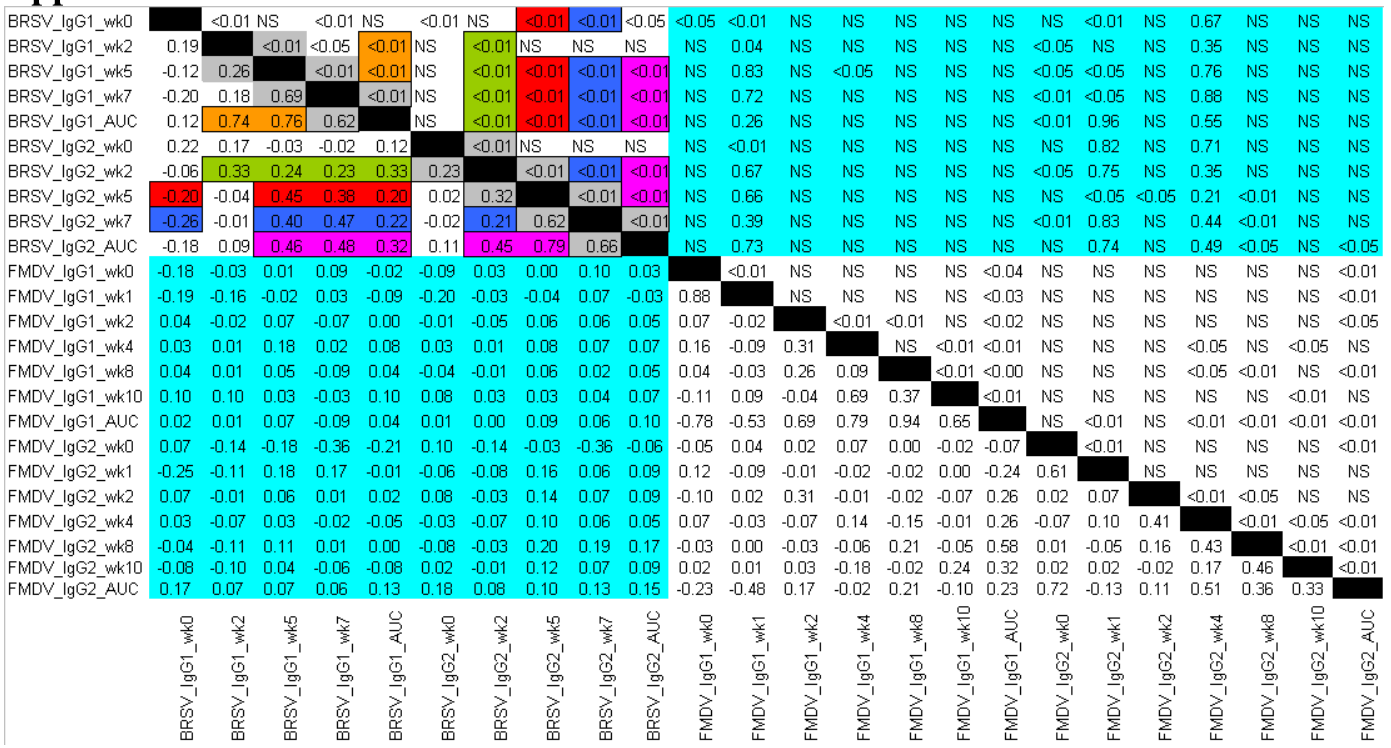
Appendix 3.1

Immunisation[1]	Trait[2]	Mean[3]	Range[4]	SD[5]
FMDV peptide - IgG Responses	IgG1_week_0	1.22	0.00 - 9.31	1.36
	IgG1_week_1	1.33	0.00 - 8.86	1.402
	IgG1_week_2	69.74	0.00 - 322.86	66.16
	IgG1_week_4	166.95	0.00 - 672.03	144.39
	IgG1_week_8	291.65	0.00 - 1254.56	269.20
	IgG1_week_10	176.12	0.00 - 670.78	141.63
	IgG1_AUC	1658.47	0.08 - 6421.13	1381.31
	IgG2_week_0	0.01	0.00 - 0.35	0.05
	IgG2_week_1	0.01	0.00 - 0.49	0.0505
	IgG2_week_2	5.64	0.00 - 51.07	8.80
	IgG2_week_4	13.09	0.00 - 87.85	17.32
	IgG2_week_8	24.35	0.00 - 810.60	64.70
	IgG2_week_10	23.50	0.00 - 968.30	71.77
	IgG2_AUC	144.41	0.01 - 3622.06	302.30
BRSV Vaccine - IgG Responses	IgG1_week_0.	13.82	0.03 - 94.03	20.40
	IgG1_week_2.	20.03	1.24 - 93.03	15.44
	IgG1_week_5.	38.96	4.03 - 110.95	19.13
	IgG1_week_7.	31.20	1.32 - 98.77	20.31
	IgG1_AUC	191.13	10.37 - 592.77	89.66
	IgG2_week_0.	2.16	0.02 - 20.14	2.57
	IgG2_week_2.	4.54	0.05 - 30.44	4.09
	IgG2_week_5.	12.55	0.15 - 86.09	10.75
	IgG2_week_7.	13.07	0.06 - 81.03	13.23
	IgG2_AUC	57.36	0.50 - 327.39	43.15

Phenotype summaries

1. Immunisation with FMDV peptide (Chapter 1); BRSV vaccine
2. Trait: each trait is shown as follows: total IgG or IgG isotype response, followed by the week relative to vaccination.
3. Mean: mean average of each time point. BRSV measured in Optical Density whilst FMDV is measured in µl/ml.
4. Range: Minimum and maximum response for each time point (units as 3.).
5. SD: the standard deviation of each trait mean at each time point

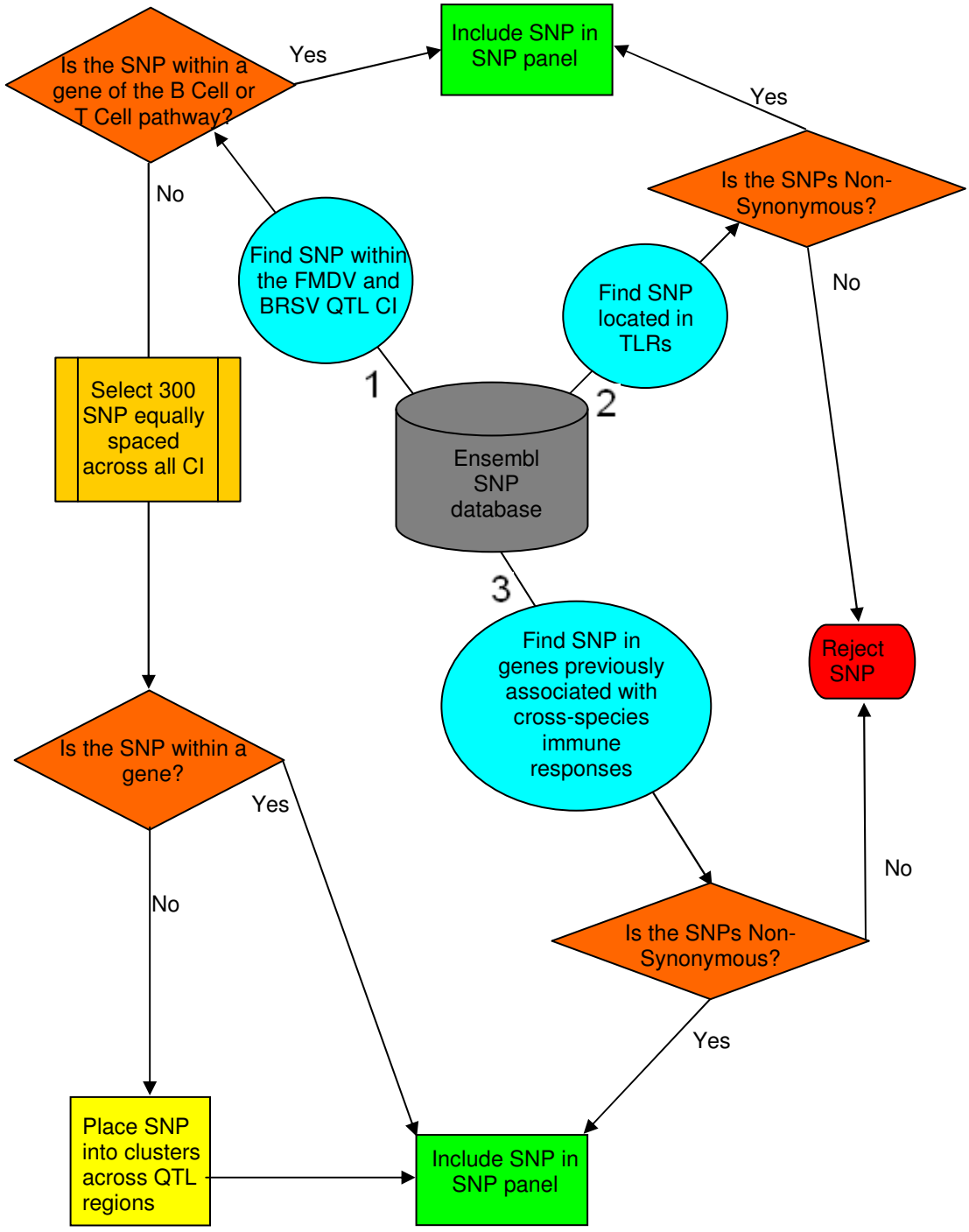
Appendix 3.2



	BRSV_IgG1_wk0	BRSV_IgG1_wk2	BRSV_IgG1_wk5	BRSV_IgG1_wk7	BRSV_IgG1_AUC	BRSV_IgG2_wk0	BRSV_IgG2_wk2	BRSV_IgG2_wk5	BRSV_IgG2_wk7	BRSV_IgG2_AUC	FMDV_IgG1_wk0	FMDV_IgG1_wk1	FMDV_IgG1_wk2	FMDV_IgG1_wk4	FMDV_IgG1_wk8	FMDV_IgG1_wk10	FMDV_IgG1_AUC	FMDV_IgG2_wk0	FMDV_IgG2_wk1	FMDV_IgG2_wk2	FMDV_IgG2_wk4	FMDV_IgG2_wk8	FMDV_IgG2_wk10	FMDV_IgG2_AUC
BRSV_IgG1_wk0		<0.01	NS	<0.01	NS	<0.01	NS	<0.01	<0.01	<0.05	<0.05	<0.01	NS	NS	NS	NS	NS	NS	<0.01	NS	0.67	NS	NS	NS
BRSV_IgG1_wk2	0.19		<0.01	<0.05	<0.01	NS	<0.01	NS	NS	NS	NS	0.04	NS	NS	NS	NS	NS	<0.05	NS	NS	0.35	NS	NS	NS
BRSV_IgG1_wk5	-0.12	0.26		<0.01	<0.01	NS	<0.01	<0.01	<0.01	<0.01	NS	0.83	NS	<0.05	NS	NS	NS	<0.05	<0.05	NS	0.76	NS	NS	NS
BRSV_IgG1_wk7	-0.20	0.18	0.69		<0.01	NS	<0.01	<0.01	<0.01	<0.01	NS	0.72	NS	NS	NS	NS	NS	<0.01	<0.05	NS	0.88	NS	NS	NS
BRSV_IgG1_AUC	0.12	0.74	0.76	0.62		NS	<0.01	<0.01	<0.01	<0.01	NS	0.26	NS	NS	NS	NS	NS	<0.01	0.96	NS	0.55	NS	NS	NS
BRSV_IgG2_wk0	0.22	0.17	-0.03	-0.02	0.12		<0.01	NS	NS	NS	NS	<0.01	NS	NS	NS	NS	NS	NS	0.82	NS	0.71	NS	NS	NS
BRSV_IgG2_wk2	-0.06	0.33	0.24	0.23	0.33	0.23		<0.01	<0.01	<0.01	NS	0.67	NS	NS	NS	NS	NS	<0.05	0.75	NS	0.35	NS	NS	NS
BRSV_IgG2_wk5	-0.20	-0.04	0.45	0.38	0.20	0.02	0.32		<0.01	<0.01	NS	0.66	NS	NS	NS	NS	NS	NS	<0.05	<0.05	0.21	<0.01	NS	NS
BRSV_IgG2_wk7	-0.26	-0.01	0.40	0.47	0.22	-0.02	0.21	0.62		<0.01	NS	0.39	NS	NS	NS	NS	NS	<0.01	0.83	NS	0.44	<0.01	NS	NS
BRSV_IgG2_AUC	-0.18	0.09	0.46	0.48	0.32	0.11	0.45	0.79	0.66		NS	0.73	NS	NS	NS	NS	NS	NS	0.74	NS	0.49	<0.05	NS	<0.05
FMDV_IgG1_wk0	-0.18	-0.03	0.01	0.09	-0.02	-0.09	0.03	0.00	0.10	0.03		<0.01	NS	NS	NS	NS	<0.04	NS	NS	NS	NS	NS	NS	<0.01
FMDV_IgG1_wk1	-0.19	-0.16	-0.02	0.03	-0.09	-0.20	-0.03	-0.04	0.07	-0.03	0.88		NS	NS	NS	NS	<0.03	NS	NS	NS	NS	NS	NS	<0.01
FMDV_IgG1_wk2	0.04	-0.02	0.07	-0.07	0.00	-0.01	-0.05	0.06	0.06	0.05	0.07	-0.02		<0.01	<0.01	NS	<0.02	NS	NS	NS	NS	NS	NS	<0.05
FMDV_IgG1_wk4	0.03	0.01	0.18	0.02	0.08	0.03	0.01	0.08	0.07	0.07	0.16	-0.09	0.31		NS	<0.01	<0.01	NS	NS	NS	<0.05	NS	<0.05	NS
FMDV_IgG1_wk8	0.04	0.01	0.05	-0.09	0.04	-0.04	-0.01	0.06	0.02	0.05	0.04	-0.03	0.26	0.09		<0.01	<0.00	NS	NS	NS	<0.05	<0.01	NS	<0.01
FMDV_IgG1_wk10	0.10	0.10	0.03	-0.03	0.10	0.08	0.03	0.03	0.04	0.07	-0.11	0.09	-0.04	0.69	0.37		<0.01	NS	NS	NS	NS	<0.01	NS	
FMDV_IgG1_AUC	0.02	0.01	0.07	-0.09	0.04	0.01	0.00	0.09	0.06	0.10	-0.78	-0.53	0.69	0.79	0.94	0.65		NS	<0.01	NS	<0.01	<0.01	<0.01	<0.01
FMDV_IgG2_wk0	0.07	-0.14	-0.18	-0.36	-0.21	0.10	-0.14	-0.03	-0.36	-0.06	-0.05	0.04	0.02	0.07	0.00	-0.02	-0.07		<0.01	NS	NS	NS	NS	<0.01
FMDV_IgG2_wk1	-0.25	-0.11	0.18	0.17	-0.01	-0.06	-0.08	0.16	0.06	0.09	0.12	-0.09	-0.01	-0.02	-0.02	0.00	-0.24	0.61		NS	NS	NS	NS	NS
FMDV_IgG2_wk2	0.07	-0.01	0.06	0.01	0.02	0.08	-0.03	0.14	0.07	0.09	-0.10	0.02	0.31	-0.01	-0.02	-0.07	0.26	0.02	0.07		<0.01	<0.05	NS	NS
FMDV_IgG2_wk4	0.03	-0.07	0.03	-0.02	-0.05	-0.03	-0.07	0.10	0.06	0.05	0.07	-0.03	-0.07	0.14	-0.15	-0.01	0.26	-0.07	0.10	0.41		<0.01	<0.05	<0.01
FMDV_IgG2_wk8	-0.04	-0.11	0.11	0.01	0.00	-0.08	-0.03	0.20	0.19	0.17	-0.03	0.00	-0.03	-0.06	0.21	-0.05	0.58	0.01	-0.05	0.16	0.43		<0.01	<0.01
FMDV_IgG2_wk10	-0.08	-0.10	0.04	-0.06	-0.08	0.02	-0.01	0.12	0.07	0.09	0.02	0.01	0.03	-0.18	-0.02	0.24	0.32	0.02	0.02	-0.02	0.17	0.46		<0.01
FMDV_IgG2_AUC	0.17	0.07	0.07	0.06	0.13	0.18	0.08	0.10	0.13	0.15	-0.23	-0.48	0.17	-0.02	0.21	-0.10	0.23	0.72	-0.13	0.11	0.51	0.36	0.33	

Correlations within and between the immune responses to the BRSV vaccine and the FMDV peptide.
The x and y Axis contain each week of the BRSV and the FMDV study [155] with correlations located below the black diagonal line. Above the black diagonal line is the significance of each correlation. The large blue boxes highlight the correlations and significance of the correlations which exist between the FMDV study and the current study. The horizontal orange (IgG1 AUC measurement), green (IgG2 levels two post vaccination), red (IgG2 levels 5 weeks post vaccination), blue (IgG2 levels 7 weeks post vaccination) and purple (IgG2 AUC measurement) lines represent the highly significant correlations throughout the BRSV study. The vertical coloured lines highlight the corresponding significance of correlations.

Appendix 4.1



Flow chart explaining the SNP selection criteria
The process begins in the centre of the diagram, at the Ensembl SNP data base. From the centre, 3 searches occur (blue circles). From the search results further refining of potential SNPs occurs in the orange diamonds. Thus, SNP are accepted into the SNP panel (green rectangles), rejected (red oval) or have to be further refined (yellow squares) before being accepted into the SNP panel.

Appendix 4.2

```
#!/usr/bin/perl

use strict ;
use warnings ;
use Getopt::Long;
use Env qw( HOME);

use lib ("${HOME}/src/bioperl-live");
use lib ("${HOME}/src/ensembl/modules");
use lib ("${HOME}/src/ensembl-compara/modules");
use lib ("${HOME}/src/ensembl-external/modules");
use lib ("${HOME}/src/ensembl-functgenomics/modules");
use lib ("${HOME}/src/ensembl-variation/modules");
use Bio::EnsEMBL::Registry;

die "No species supplied\n" unless $ARGV[0] =~ m/^cow|pig|sheep|human|chicken$/i;

my $registry = "Bio::EnsEMBL::Registry";

$registry->load_registry_from_db(
  -host => 'ensembldb.ensembl.org',
  -user => 'anonymous'
  );
print "Connection made...\n";
my $slice_adaptor = $registry->get_adaptor( $ARGV[0], 'Core', 'Slice' );
my $tr_adaptor = $registry->get_adaptor( $ARGV[0], 'Core', 'Transcript' );
my $daf_adaptor = $registry->get_adaptor( $ARGV[0], 'Core', 'DnaAlignFeature' );
my $species = "";


open (IN, "$ARGV[1]");
while (my $chr = <IN>) {
  chomp ($chr);
  my $start = <IN>;
  chomp ($start);
  my $end = <IN>;
  chomp ($end);

my $slice = $slice_adaptor->fetch_by_region( 'chromosome', $chr, $start, $end );
      my $transcripts = $tr_adaptor->fetch_all_by_Slice($slice);
      while ( my $tr = shift @{$transcripts} ) {
        my $dbID = $tr->dbID();
        my $start = $tr->start();
        my $end = $tr->end();
        my $strand = $tr->strand();
        my $stable_id = $tr->stable_id();
        print join( "\t", $chr, $stable_id, $dbID, $start, $end, $strand ), "\n";
      }
}
```

Perl Script
Perl script used to access Ensembl and retrieve SNPs

Appendix 4.3

Chromosome[1]	SNP name[2]	Position[3]	Status[4]
2	rs43282727	1,145,842	Success
2	rs42368097	3,372,686	Fail
2	rs43099540	4,145,241	Fail
2	rs43288056	4,526,658	Success
2	rs43287110	5,009,410	Fail
2	rs43284763	5,385,388	Fail
2	rs43287124	6,173,061	Fail
2	rs43283828	7,758,030	Fail
2	rs42271298	8,547,313	Success
2	rs43289279	9,577,829	Success
2	rs43287355	10,408,218	Fail
2	rs42534616	14,113,356	Fail
2	rs42300304	14,868,782	Success
2	rs42239339	15,119,485	Fail
2	rs42239334	15,132,187	Fail
2	rs43291704	18,283,013	Success
2	rs43293712	21,407,841	Fail
2	rs43297955	34,981,686	Success
2	rs42710296	37,975,105	Fail
2	rs43306573	43,161,115	Fail
2	rs42557939	44,190,522	Fail
2	rs43325273	125,502,733	Fail
3	rs42447625	64,189,555	Fail
3	rs43346321	65,229,353	Success
3	rs42820431	82,342,593	Success
3	rs42223380	82,660,195	Success
4	rs42661063	4,872,040	Fail
4	rs41811275	7,533,744	Success
4	rs41848377	9,040,359	Success
4	rs43372164	10,064,006	Success
4	rs42555252	11,583,413	Success
4	rs43158855	12,698,631	Fail
4	rs43376075	15,979,413	Fail
4	rs43380234	19,482,457	Success
4	rs43372439	21,486,169	Success
4	rs43379261	26,101,583	Fail
4	rs42628233	27,701,164	Success
4	rs43062765	31,522,127	Success
4	rs42601449	31,855,593	Fail
4	rs43380660	32,752,933	Success
4	rs43706506	32,760,739	Fail
4	rs43396806	45,828,047	Success
4	rs43397964	45,839,476	Success
4	rs43410172	77,534,161	Success
4	rs17870216	78,900,409	Fail
5	rs29023087	64,857,106	Fail
5	rs43434899	67,635,108	Fail
6	rs42688655	230,007	Success
6	rs42578633	531,485	Success
6	rs29022925	2,212,858	Success

6	rs42579196	2,255,433	Success
6	rs42765470	10,812,211	Success
6	rs42976134	12,632,047	Success
6	rs43450138	13,225,484	Success
6	rs43451327	13,429,185	Fail
6	rs43455095	23,422,841	Success
6	rs43457496	23,575,406	Fail
6	rs43457274	26,738,103	Fail
6	SNP20_ADH7_b1_224C_T_GQ	27,088,618	Success
6	rs42573616	28,467,373	Fail
6	rs29026551	36,787,039	Success
6	SNP36_BTA-75976	36,848,688	Success
6	SNP37_BTA-75814	37,231,101	Fail
6	SNP01_ABCG2_int_29444	37,384,988	Fail
6	SNP57_ABCG2_256_Olsen2007	37,418,728	Fail
6	SNP06_OPN_11740_AC_Sch	37,512,064	Fail
6	SNP07_OPN_ex7_156531_CohZ	37,512,073	Success
6	SNP05_OPN_10043_TC_Sch	37,513,761	Success
6	SNP04_OPN_5896_GA_Sch	37,517,911	Fail
6	SNP03_OPN_5075_CT_Sch	37,518,733	Fail
6	SNP02_OPN_3492_AG_Schn	37,520,315	Fail
6	SNP26_OPN_1406_TC_Schn	37,522,401	Success
6	SNP33_IBSP_exon7_802post	37,681,944	Success
6	SNP38_BTA-75780	37,689,720	Fail
6	rs43455199	37,703,987	Fail
6	SNP08_MEPE_int2_151126166	37,704,773	Fail
6	SNP62_NCAPG_exon9Ile442Met	38,164,403	Fail
6	SNP42_BTA-23366	38,876,957	Fail
6	SNP43_BTA-23368	39,030,695	Success
6	SNP44_BTA-75882	39,748,005	Success
6	SNP45_BTA-114459	40,049,321	Success
6	SNP39_BTA-75889	41,077,118	Success
6	rs42852239	41,352,734	Fail
6	SNP61_KCNIP4_SNP09_exon3	41,431,834	Success
6	SNP60_KCNIP4_SNP08_exon3	41,431,873	Success
6	SNP59_KCNIP4_SNP02_intron1	41,439,745	Success
6	SNP58_KCNIP4_SNP01_exon1	41,522,884	Success
6	SNP40_BTA-75916	41,674,742	Success
6	SNP41_BTA-75900	41,867,013	Success
6	SNP46_BTA-75936	42,512,116	Success
6	SNP47_BTA-08168	43,365,830	Fail
6	rs43702384	43,533,567	Success
6	SNP48_BTA-97410	44,180,262	Success
6	SNP49_BTA-76032	44,670,127	Success
6	SNP16_PPARGC1A_3UTR_5314	44,733,159	Success
6	SNP14_PPARGC1A_3UTR_3359	44,735,115	Success
6	SNP13_PPARGC1A_in9_1892_19	44,753,377	Success
6	SNP12_PPARGC1A_ex9_1847	44,753,441	Success
6	SNP11_PPARGC1A_ex8_1209	44,754,643	Success
6	SNP17_PPARGC1A_BTA76037	44,768,150	Success
6	SNP18_PPARGC1A_BTA_76036	44,768,513	Success
6	SNP10_PPARGC1A_intI_49-9	44,833,216	Success

6	SNP09_PPARGC1A_prom_-920	44,839,533	Success
6	rs43457945	44,843,375	Success
6	SNP50_BTA-76040	44,853,423	Fail
6	SNP51_BTA-119876	45,758,667	Fail
6	SNP52_BTA-76106	45,960,114	Success
6	SNP53_BTA-76112	46,812,852	Fail
6	SNP55_BTA-76147	48,249,827	Success
6	SNP56_BTA-02857	48,676,372	Success
6	rs55617297	60,339,445	Fail
6	rs55617311	60,340,667	Fail
6	rs55617324	60,340,745	Success
6	rs55617197	60,340,811	Fail
6	rs55617325	60,340,994	Fail
6	rs55617137	60,341,354	Fail
6	rs55617437	60,341,651	Fail
6	rs55617267	60,356,135	Success
6	rs55617317	60,356,375	Fail
6	rs55617385	60,357,533	Fail
6	rs55617254	60,357,900	Fail
6	ss104796354	60,373,429	Success
6	ss104796353	60,373,564	Fail
6	ss104796352	60,373,576	Fail
6	ss104796350	60,373,615	Success
6	ss104796347	60,373,714	Fail
6	ss104796344	60,374,010	Success
6	ss104796341	60,374,554	Fail
6	rs43702941	60,374,554	Fail
6	ss104796340	60,374,934	Fail
6	ss104796339	60,375,010	Success
6	ss104796338	60,375,065	Success
6	rs43465290	63,382,448	Success
6	rs43144881	63,856,283	Fail
7	rs42371911	372,053	Success
7	rs43494261	817,185	Success
7	rs43502716	17,900,867	Success
7	rs43502714	17,900,946	Success
7	rs43502712	17,901,772	Success
7	rs43502711	17,901,960	Success
7	rs42468294	42,159,162	Fail
7	rs42754503	42,406,483	Success
7	rs29024743	81,122,729	Success
7	rs42123134	82,574,137	Fail
8	rs43551637	40,328,094	Fail
8	rs43551679	42,212,253	Fail
8	rs43556471	62,463,474	Fail
8	rs42254196	67,719,297	Fail
8	rs43289334	89,845,143	Fail
8	rs43572154	90,426,160	Success
8	rs8193041	112,427,272	Fail
8	rs8193048	112,431,958	Fail
8	rs8193049	112,435,012	Success
8	rs8193050	112,435,274	Fail

8	rs8193053	112,435,599	Fail
8	rs8193055	112,435,702	Fail
8	rs8193066	112,436,507	Fail
8	rs8193069	112,436,580	Success
9	rs43596425	35,663,409	Success
9	rs42932713	36,045,372	Success
9	rs42472240	63,602,153	Fail
9	rs43608009	66,562,158	Fail
11	rs42312327	5,732,048	Success
11	rs43549594	6,120,325	Success
11	rs29011157	15,996,717	Success
11	rs43671260	16,899,234	Fail
12	rs43689833	3,685,550	Fail
12	rs43038502	9,310,538	Success
12	rs42420659	13,010,703	Fail
13	rs42633118	32,620,521	Success
13	rs41688025	38,310,581	Success
14	rs41716631	571,324	Fail
14	rs41716552	1,124,497	Fail
14	rs41718866	10,064,223	Success
14	rs41726305	19,210,519	Success
14	rs41722114	23,535,844	Success
15	rs41746924	14,222,942	Fail
15	rs42205048	19,855,733	Success
15	rs42309927	65,451,191	Success
16	rs55617166	23,622,625	Fail
16	rs55617270	23,623,111	Fail
16	rs55617251	23,623,174	Fail
16	rs55617330	23,623,824	Fail
16	rs55617329	23,624,016	Fail
16	rs55617168	23,624,607	Fail
16	rs43715512	46,625,896	Fail
16	rs43156472	53,793,735	Fail
16	rs41823113	64,715,652	Fail
16	rs41817317	67,077,026	Success
17	ss95214878	4,282,692	Fail
17	ss95214875	4,282,845	Success
17	ss95214874	4,282,879	Fail
17	ss95214873	4,283,027	Fail
17	ss95214869	4,283,153	Fail
17	ss95214868	4,283,336	Success
17	ss95214867	4,283,384	Fail
17	ss95214865	4,283,590	Success
17	ss95214864	4,283,627	Fail
17	ss95214863	4,283,831	Success
17	ss95214862	4,283,852	Fail
17	ss95214861	4,283,863	Fail
17	ss95214860	4,283,916	Fail
17	ss95214859	4,283,926	Fail
17	ss104796314	4,283,970	Fail
17	ss95214858	4,284,160	Fail
17	ss95214857	4,284,209	Success

17	ss95214856	4,284,373	Fail
17	ss95214855	4,284,386	Success
17	ss95214854	4,284,639	Success
17	ss104796308	4,284,651	Fail
17	ss95214853	4,284,652	Fail
17	rs41840540	18,434,183	Fail
17	rs41846165	53,639,297	Fail
17	rs41843359	59,991,168	Success
18	rs43073975	7,627,591	Fail
18	rs41861310	9,450,758	Fail
18	rs41861475	12,608,656	Fail
18	rs41871255	17,513,388	Success
18	rs41882274	42,990,977	Fail
18	rs41881585	43,008,150	Success
18	rs41883772	45,466,996	Success
18	rs41889047	45,985,711	Fail
18	rs41889048	45,992,932	Fail
18	rs17871896	55,089,314	Success
18	rs41897525	55,089,413	Success
18	rs41893756	55,201,773	Success
18	rs41893757	55,201,874	Success
18	rs41890230	55,297,550	Fail
18	rs41890229	55,299,321	Fail
18	rs41893490	55,856,642	Success
18	rs41894171	55,872,024	Success
18	rs41894194	55,914,102	Fail
18	rs41893053	56,536,828	Fail
18	rs41895599	56,762,980	Success
18	rs41895600	56,763,022	Success
18	rs41895602	56,763,691	Fail
18	rs41895604	56,763,769	Fail
18	rs41890820	57,351,399	Success
19	rs42399876	8,909,768	Fail
19	rs41903052	10,047,803	Fail
19	rs41255557	10,047,900	Success
19	rs41899079	14,643,966	Fail
19	rs41909225	17,477,209	Fail
19	rs41903246	22,710,810	Success
19	rs41909114	25,258,154	Success
19	rs41910166	28,079,648	Success
19	rs43329536	28,376,932	Success
19	rs42467700	32,953,578	Fail
19	rs41915330	36,738,471	Success
19	rs41914650	38,967,695	Success
20	rs42288095	1,670,625	Fail
20	rs43349824	4,719,720	Fail
20	rs42661323	5,043,835	Fail
20	rs42263233	5,749,563	Fail
20	rs42638947	6,387,054	Success
20	rs41977113	6,901,362	Success
20	rs42755290	7,976,253	Success
20	rs41935052	8,415,423	Success

20	rs41933917	8,434,911	Fail
20	rs41932920	8,463,797	Success
20	rs41931981	9,874,394	Fail
20	rs41931155	9,973,787	Fail
20	rs17871699	10,128,876	Success
20	rs42931384	12,933,656	Success
20	rs42819473	13,172,735	Fail
20	rs42241895	13,401,254	Success
20	rs41939013	14,562,036	Success
20	rs41939858	14,765,661	Fail
20	rs41940658	14,931,073	Success
20	rs41257426	15,536,536	Fail
20	rs41935901	19,180,080	Fail
20	rs41938903	21,702,493	Fail
20	rs42745029	21,946,837	Fail
20	rs41939754	24,584,116	Fail
20	rs41255556	25,605,974	Success
21	rs41971213	20,838,959	Fail
21	rs42164128	20,858,512	Success
21	rs41976145	34,904,887	Fail
21	rs29016829	35,150,184	Success
21	rs42502308	41,267,701	Success
22	rs41994846	6,078,800	Fail
22	rs41997469	8,095,553	Fail
22	rs41997472	8,116,283	Fail
22	rs17871073	11,569,447	Fail
22	rs41998546	11,627,557	Success
22	rs41995921	11,690,241	Success
22	rs41995914	11,696,113	Success
22	rs42000457	15,043,101	Fail
22	rs42655015	16,029,150	Success
22	rs55617136	49,673,859	Fail
22	rs55617258	49,674,747	Success
23	rs42022743	344,771	Fail
23	rs42022227	1,497,055	Fail
23	rs42021087	1,939,695	Fail
23	rs43707857	2,797,676	Success
23	rs42021749	3,422,699	Fail
23	rs42021755	3,550,188	Fail
23	rs42021824	3,735,697	Fail
23	rs42023442	7,255,826	Fail
23	rs43008782	7,342,237	Fail
23	rs42023448	7,368,999	Fail
23	rs43049152	8,758,710	Fail
23	rs42020089	9,344,087	Fail
23	rs42612806	9,703,201	Fail
23	rs42612805	9,710,886	Fail
23	rs42025129	10,147,019	Fail
23	rs43706486	10,330,943	Success
23	rs42022558	12,548,875	Fail
23	rs41255662	12,989,057	Fail
23	rs42451594	13,659,714	Fail

23	rs42451592	13,675,312	Fail
23	rs41257079	15,615,455	Fail
23	rs42021406	15,702,335	Fail
23	rs42021387	16,002,474	Fail
23	rs42027576	17,166,685	Fail
23	rs42027575	17,172,021	Fail
23	rs42026557	18,385,689	Fail
23	rs42025725	18,396,766	Fail
23	rs43054367	19,024,064	Fail
23	rs41255407	19,886,383	Fail
23	rs42026571	20,104,460	Fail
23	rs42027749	20,521,898	Fail
23	rs42541025	21,192,214	Fail
23	rs43703954	22,575,043	Success
23	rs42028278	23,813,406	Fail
23	rs42028267	23,880,248	Success
23	rs42027020	25,004,112	Fail
23	rs42028329	25,399,173	Fail
23	rs29015593	25,583,126	Success
23	rs42311145	26,280,492	Fail
23	rs42311136	26,280,557	Fail
23	rs42023981	27,021,896	Fail
23	rs42023980	27,124,500	Fail
23	rs42032378	27,434,885	Fail
23	rs42032371	27,444,055	Fail
23	rs17870353	27,444,757	Fail
23	rs42032370	27,451,908	Fail
23	rs41642092	27,541,569	Fail
23	rs41587590	27,731,019	Success
23	rs17871955	28,074,549	Fail
23	rs42579977	28,684,909	Fail
23	rs42580008	28,687,100	Fail
23	rs42024011	28,895,621	Fail
23	rs41257015	28,965,211	Success
23	rs42029155	29,328,304	Fail
23	rs42030040	29,763,471	Fail
23	rs43030763	29,940,497	Success
23	rs29017177	30,273,732	Success
23	rs29015191	30,502,689	Success
23	rs43350855	30,853,358	Fail
23	rs43708439	31,601,937	Success
23	rs42030070	32,010,522	Fail
23	rs42851411	32,187,753	Fail
23	rs41257282	33,221,160	Success
23	rs41699902	33,833,542	Fail
23	rs41666288	34,809,053	Fail
23	rs41666290	34,949,047	Fail
23	rs43160956	35,060,107	Fail
23	rs42027035	35,951,533	Fail
23	rs42372315	37,855,828	Fail
23	rs43705168	38,240,446	Success
23	rs43281402	39,589,320	Fail

23	rs42109669	39,771,140	Fail
23	rs42027059	39,879,827	Fail
23	rs29021595	40,284,987	Success
23	rs42027176	40,604,396	Fail
23	rs42027632	41,547,061	Fail
23	rs42027230	41,567,237	Fail
23	rs41257112	42,049,908	Success
23	rs42032036	43,227,492	Success
23	rs41743748	43,368,217	Success
23	rs41256961	43,476,116	Success
23	rs42029541	44,923,043	Success
23	rs42029586	44,959,165	Success
23	rs42028513	45,489,977	Success
23	rs42034874	45,779,639	Fail
23	rs42342086	46,094,090	Success
23	rs42342962	46,182,144	Success
23	rs42345438	46,401,034	Success
24	rs41805428	20,552,592	Fail
24	rs42041883	24,415,723	Fail
24	rs42819260	26,689,961	Fail
24	rs42048416	27,077,243	Fail
24	rs41257081	27,130,822	Success
24	rs42049356	29,741,673	Success
24	rs42045874	30,519,872	Fail
24	rs42042620	30,796,671	Success
24	rs42441026	31,550,540	Fail
24	rs42053876	38,815,332	Success
24	rs42054102	43,464,945	Fail
25	rs42057017	20,719,152	Fail
25	rs42057705	22,152,954	Fail
25	rs43695847	22,178,379	Fail
25	rs42060100	23,945,464	Success
25	rs42058469	24,157,508	Fail
25	rs42061802	26,534,002	Fail
25	rs42074207	27,954,457	Fail
25	rs42068459	28,404,390	Fail
25	rs42690103	30,915,753	Success
25	rs42068744	34,696,283	Success
25	rs42068083	34,703,184	Fail
25	rs42070647	34,829,906	Success
26	rs42102914	36,858,669	Success
26	rs42631147	38,247,006	Fail
27	rs42544474	348,147	Success
27	rs42851916	17,492,663	Fail
27	rs55617204	17,497,632	Success
27	rs55617272	17,497,892	Fail
27	rs42852439	17,498,607	Success
27	rs42118884	21,956,554	Fail
27	rs43708798	23,118,868	Success
27	rs43731750	35,329,115	Fail
27	rs43729240	36,428,655	Fail
29	rs42978304	288,597	Fail

	29	rs42155456	574,482	Success
	29	rs42189764	43,688,138	Fail
X		rs55617390	82,015,995	Success
X		rs55617351	82,016,881	Success
X		rs55617145	82,017,087	Success
X		rs55617174	82,017,180	Success
X		rs55617354	82,017,265	Success
X		rs55617433	82,120,395	Fail
X		rs55617309	82,121,227	Fail
X		rs55617183	82,122,372	Success
X		rs55617320	82,122,592	Fail

All SNPs selected for genotyping.

1. Chromosome the SNP is located on.
2. The common name of the SNP.
3. The position (Base Pairs) of the SNP along its chromosome.
4. Status of the SNP: Success = genotyped successfully on the F2 and backcross of the RoBoGen herd, Fail=not successfully genotyped.

Appendix 4.4

Chromosome[1]	SNP name[2]	Position[3]	Placement Type[4]	Status[5]
2	rs43282727	1,145,842	1	1
2	rs43288056	4,526,658	1	1
2	rs42271298	8,547,313	1	1
2	rs43289279	9,577,829	1	1
2	rs42300304	14,868,782	1	1
2	rs43291704	18,283,013	1	1
2	rs43297955	34,981,686	1	2
3	rs43346321	65,229,353	1	3
3	rs42820431	82,342,593	1	1
3	rs42223380	82,660,195	1	1
4	rs41811275	7,533,744	1	1
4	rs41848377	9,040,359	1	1
4	rs43372164	10,064,006	1	1
4	rs42555252	11,583,413	1	1
4	rs43380234	19,482,457	1	1
4	rs43372439	21,486,169	1	1
4	rs42628233	27,701,164	1	1
4	rs43062765	31,522,127	1	1
4	rs43380660	32,752,933	1	1
4	rs43396806	45,828,047	1	1
4	rs43397964	45,839,476	1	1
4	rs43410172	77,534,161	1	1
6	rs42688655	230,007	1	1
6	rs42578633	531,485	1	2
6	rs29022925	2,212,858	1	1
6	rs42579196	2,255,433	1	1
6	rs42765470	10,812,211	1	1
6	rs42976134	12,632,047	1	1
6	rs43450138	13,225,484	1	2
6	rs43455095	23,422,841	1	1
6	SNP20_ADH7_b1_224C_T_GQ	27,088,618	1	1
6	rs29026551	36,787,039	1	1
6	SNP36_BTA-75976	36,848,688	1	1
6	SNP07_OPN_ex7_156531_CohZ	37,512,073	1	1
6	SNP05_OPN_10043_TC_Sch	37,513,761	1	1
6	SNP26_OPN_1406_TC_Schn	37,522,401	1	1
6	SNP33_IBSP_exon7_802post	37,681,944	1	2
6	SNP43_BTA-23368	39,030,695	1	1
6	SNP44_BTA-75882	39,748,005	1	1
6	SNP45_BTA-114459	40,049,321	1	3
6	SNP39_BTA-75889	41,077,118	1	1
6	SNP61_KCNIP4_SNP09_exon3	41,431,834	1	1
6	SNP60_KCNIP4_SNP08_exon3	41,431,873	1	1
6	SNP59_KCNIP4_SNP02_intron1	41,439,745	1	1
6	SNP58_KCNIP4_SNP01_exon1	41,522,884	1	1
6	SNP40_BTA-75916	41,674,742	1	1
6	SNP41_BTA-75900	41,867,013	1	1
6	SNP46_BTA-75936	42,512,116	1	2
6	rs43702384	43,533,567	1	1

6	SNP48_BTA-97410	44,180,262	1	2
6	SNP49_BTA-76032	44,670,127	1	1
6	SNP16_PPARGC1A_3UTR_5314	44,733,159	1	1
6	SNP14_PPARGC1A_3UTR_3359	44,735,115	1	3
6	SNP13_PPARGC1A_in9_1892_19	44,753,377	1	2
6	SNP12_PPARGC1A_ex9_1847	44,753,441	1	1
6	SNP11_PPARGC1A_ex8_1209	44,754,643	1	1
6	SNP17_PPARGC1A_BTA76037	44,768,150	1	1
6	SNP18_PPARGC1A_BTA_76036	44,768,513	1	2
6	SNP10_PPARGC1A_intI_49-9	44,833,216	1	1
6	SNP09_PPARGC1A_prom_-920	44,839,533	1	1
6	rs43457945	44,843,375	1	1
6	SNP52_BTA-76106	45,960,114	1	1
6	SNP55_BTA-76147	48,249,827	1	2
6	SNP56_BTA-02857	48,676,372	1	2
6	rs55617324	60,340,745	2	2
6	rs55617267	60,356,135	2	2
6	ss104796354	60,373,429	2	2
6	ss104796350	60,373,615	2	1
6	ss104796344	60,374,010	2	1
6	ss104796339	60,375,010	2	1
6	ss104796338	60,375,065	2	1
6	rs43465290	63,382,448	1	1
7	rs42371911	372,053	1	1
7	rs43494261	817,185	1	1
7	rs43502716	17,900,867	1	3
7	rs43502714	17,900,946	1	2
7	rs43502712	17,901,772	1	1
7	rs43502711	17,901,960	1	1
7	rs42754503	42,406,483	1	1
7	rs29024743	81,122,729	1	2
8	rs43572154	90,426,160	1	2
8	rs8193049	112,435,012	2	1
8	rs8193069	112,436,580	2	1
9	rs43596425	35,663,409	1	1
9	rs42932713	36,045,372	1	1
11	rs42312327	5,732,048	1	1
11	rs43549594	6,120,325	1	1
11	rs29011157	15,996,717	1	1
12	rs43038502	9,310,538	1	1
13	rs42633118	32,620,521	1	2
13	rs41688025	38,310,581	1	1
14	rs41718866	10,064,223	1	1
14	rs41726305	19,210,519	1	3
14	rs41722114	23,535,844	1	1
15	rs42205048	19,855,733	1	1
15	rs42309927	65,451,191	1	1
16	rs41817317	67,077,026	1	2
17	ss95214875	4,282,845	2	1
17	ss95214868	4,283,336	2	1
17	ss95214865	4,283,590	2	1
17	ss95214863	4,283,831	2	2

17	ss95214857	4,284,209	2	3
17	ss95214855	4,284,386	2	1
17	ss95214854	4,284,639	2	1
17	rs41843359	59,991,168	1	3
18	rs41871255	17,513,388	1	2
18	rs41881585	43,008,150	1	1
18	rs41883772	45,466,996	1	3
18	rs17871896	55,089,314	1	1
18	rs41897525	55,089,413	1	1
18	rs41893756	55,201,773	1	1
18	rs41893757	55,201,874	1	2
18	rs41893490	55,856,642	1	1
18	rs41894171	55,872,024	1	2
18	rs41895599	56,762,980	1	1
18	rs41895600	56,763,022	1	1
18	rs41890820	57,351,399	1	2
19	rs41255557	10,047,900	1	1
19	rs41903246	22,710,810	1	1
19	rs41909114	25,258,154	1	1
19	rs41910166	28,079,648	1	1
19	rs43329536	28,376,932	1	1
19	rs41915330	36,738,471	1	2
19	rs41914650	38,967,695	1	1
20	rs42638947	6,387,054	1	1
20	rs41977113	6,901,362	1	1
20	rs42755290	7,976,253	1	1
20	rs41935052	8,415,423	1	1
20	rs41932920	8,463,797	1	1
20	rs17871699	10,128,876	1	1
20	rs42931384	12,933,656	1	1
20	rs42241895	13,401,254	1	1
20	rs41939013	14,562,036	1	1
20	rs41940658	14,931,073	1	1
20	rs41255556	25,605,974	1	1
21	rs42164128	20,858,512	1	1
21	rs29016829	35,150,184	1	1
21	rs42502308	41,267,701	1	1
22	rs41998546	11,627,557	1	1
22	rs41995921	11,690,241	1	1
22	rs41995914	11,696,113	1	1
22	rs42655015	16,029,150	1	1
22	rs55617258	49,674,747	2	1
23	rs43707857	2,797,676	1	2
23	rs43706486	10,330,943	1	1
23	rs43703954	22,575,043	1	3
23	rs42028267	23,880,248	1	3
23	rs29015593	25,583,126	1	3
23	rs41587590	27,731,019	1	3
23	rs41257015	28,965,211	1	1
23	rs43030763	29,940,497	1	1
23	rs29017177	30,273,732	1	1
23	rs29015191	30,502,689	1	1

23	rs43708439	31,601,937	1	1
23	rs41257282	33,221,160	1	1
23	rs43705168	38,240,446	1	1
23	rs29021595	40,284,987	1	1
23	rs41257112	42,049,908	1	1
23	rs42032036	43,227,492	1	1
23	rs41743748	43,368,217	1	1
23	rs41256961	43,476,116	1	1
23	rs42029541	44,923,043	1	1
23	rs42029586	44,959,165	1	1
23	rs42028513	45,489,977	1	1
23	rs42342086	46,094,090	1	2
23	rs42342962	46,182,144	1	1
23	rs42345438	46,401,034	1	1
24	rs41257081	27,130,822	1	1
24	rs42049356	29,741,673	1	1
24	rs42042620	30,796,671	1	1
24	rs42053876	38,815,332	1	1
25	rs42060100	23,945,464	1	1
25	rs42690103	30,915,753	1	2
25	rs42068744	34,696,283	1	1
25	rs42070647	34,829,906	1	1
26	rs42102914	36,858,669	1	1
27	rs42544474	348,147	1	2
27	rs55617204	17,497,632	2	1
27	rs42852439	17,498,607	2	1
27	rs43708798	23,118,868	1	1
29	rs42155456	574,482	1	1
X	rs55617390	82,015,995	2	1
X	rs55617351	82,016,881	2	1
X	rs55617145	82,017,087	2	1
X	rs55617174	82,017,180	2	2
X	rs55617354	82,017,265	2	1
X	rs55617183	82,122,372	2	1

SNP analysed in chapter 4

Listed are all the SNP that passed all quality control measures (see Materials and Methods). The highlighted SNPs were found significant in the current study.

1. Chromosome: The chromosome the SNP is located on.
2. SNP name: The name of each SNP. Those without the standard 'RS' or 'SS'numbers have been used from *Gutierrez-Gil et al* [228].
3. Position: The position of SNPs. Measured in Base Pairs (bp).
4. Placement Type: Methodology of placing the SNP:
   (1) SNP positioned within the confidence intervals of the previous *Leach et al.* studies [155, 177], (Chapters 2 and 3).
   (2) SNP located in either TLR genes or SNP positioned in the RoBoGen herd by *Gutierrez-Gil et al* [228].
5. Status: Which phenotypes the SNPs were significantly associated with:
   (1) Both FMDV and BRSV phenotypes.
   (2) BRSV phenotypes only.
   (3) FMDV phenotypes only.

Appendix 4.5

Chromosome name[1]	Reference ID[2]	Allele[3]	Gene_ID[4]
6	rs43144881	T/A	ATP8A1
6	ss73689504	A/G	TLR1
6	ss73689408	A/G	TLR1
6	ss73689412	A/G	TLR1
6	ss73689415	A/G	TLR1
6	ss73689416	A/G	TLR1
6	ss73689466	T/C	TLR10
6	ss73689467	G/A	TLR10
6	ss73689468	C/A	TLR10
6	ss73689469	C/A	TLR10
6	ss73689470	C/G	TLR10
6	ss73689474	T/C	TLR10
6	ss73689475	A/T	TLR10
6	ss73689477	A/T	TLR10
6	ss73689478	A/G	TLR10
6	ss73689479	A/G	TLR10
6	ss73689485	A/C	TLR10
6	ss73689486	G/A	TLR10
6	ss104796338	T/G	TLR6
6	ss104796339	A/G	TLR6
6	ss104796340	A/G	TLR6
6	ss104796344	A/G	TLR6
6	ss104796347	T/A	TLR6
6	ss104796350	C/T	TLR6
6	ss104796352	C/G	TLR6
6	ss104796353	G/A	TLR6
6	ss104796354	G/A	TLR6
6	ss104796356	A/G	TLR6
6	rs43702941	G/A	TLR6
7	rs43502709	T/G	TICAM1
7	rs43502716	G/A	TICAM1
7	rs43502712	G/T	TICAM1
7	rs43502707	G/A	TICAM1
7	rs43502717	G/C	TICAM1
7	rs43502711	A/G	TICAM1
7	rs43502718	C/T	TICAM1
7	rs43502714	T/C	TICAM1
8	rs8193041	(C/T)	TLR4
8	rs8193048	(G/A)	TLR4
8	rs8193049	(A/C)	TLR4
8	rs8193050	(C/G)	TLR4
8	rs8193053	(C/A)	TLR4
8	rs8193055	A/G)	TLR4
8	rs8193066	(G/A)	TLR4
8	rs8193069	(C/T)	TLR4
16	ss73689445	A/G	TLR5
16	ss73689447	A/G	TLR5
16	ss73689448	T/C	TLR5
16	ss73689451	G/A	TLR5

16	ss73689452	C/G	TLR5
16	ss73689455	G/A	TLR5
17	ss104796308	T/C	TLR2
17	ss95214853	G/T	TLR2
17	ss95214854	G/A	TLR2
17	ss95214855	G/A	TLR2
17	ss95214856	T/G	TLR2
17	ss95214857	G/A	TLR2
17	ss95214858	A/T	TLR2
17	ss104796314	T/G	TLR2
17	ss95214859	A/C	TLR2
17	ss95214860	G/A	TLR2
17	ss95214861	T/A	TLR2
17	ss95214862	G/A	TLR2
17	ss95214863	A/G	TLR2
17	ss95214864	C/T	TLR2
17	ss95214865	A/G	TLR2
17	ss95214867	G/A	TLR2
17	ss95214868	T/G	TLR2
17	ss95214869	G/A	TLR2
17	ss95214873	C/T	TLR2
17	ss95214874	G/T	TLR2
17	ss95214875	C/G	TLR2
17	ss95214878	C/A	TLR2
17	ss104796336	G/A	TLR2
18	rs41895599	C/T	AC010325.7-202
18	rs41895600	G/A	AC010325.7-202
18	rs41895602	C/G	AC010325.7-202
18	rs41895604	G/A	AC010325.7-202
18	rs41894171	C/T	ALDH16A1
18	rs41889048	C/T	APLP1
18	rs41889051	T/C	APLP1
18	rs41883772	T/C	CD22
18	rs41894194	C/G	FCGRT
18	rs41893755	G/A	FUT1
18	rs41893756	A/G	FUT1
18	rs41893757	C/T	FUT1
18	rs41882274	C/G	GPATCH1
18	rs41881585	T/C	GPATCH1
18	rs41889047	G/T	KIRREL2
18	rs41893053	T/A	MYBPC2
18	rs41890820	C/T	NKG7
18	rs41891067	T/C	NUCB1
18	rs41891068	G/T	NUCB1
18	rs41893490	T/G	PIH1D1
18	rs41890230	T/G	PPP1R15A
18	rs41890229	T/A	PPP1R15A
18	rs17871896	C/T	SPHK2
18	rs41897525	G/T	SPHK2
22	rs42655015	G/A	ABHD5
22	rs41998546	G/A	DLEC1
22	rs41995921	C/T	DLEC1

22	rs41995914	C/G	DLEC1
22	rs41995915	G/A	DLEC1
22	rs41997469	T/A	PDCD6IP
22	rs41997472	G/T	PDCD6IP
22	rs41994846	C/G	STT3B
22	ss69357334	C/T	TLR9
22	ss69357336	G/A	TLR9
22	rs17871073	C/T	VILL
22	rs42000457	C/A	VIPR1
27	rs42851916	T/A	TLR3
27	rs55617204	A/G	TLR3
27	rs55617272	G/A	TLR3
27	rs42852439	G/T	TLR3
X	ss69357304	C/T	TLR7
X	ss69357305	G/A	TLR7
X	ss69357306	A/C	TLR7
X	ss69357307	G/A	TLR7
X	ss69357308	G/A	TLR7
X	ss69357309	G/A	TLR7
X	ss69357318	T/C	TLR8
X	ss69357319	A/T	TLR8
X	ss69357320	A/C	TLR8
X	ss69357321	C/A	TLR8
X	ss69357322	G/A	TLR8
X	ss69357323	G/A	TLR8
X	ss69357324	C/A	TLR8

SNPs used in TLRs and signalling pathways

1. Chromosome name: Chromosome the SNP marker is positioned on.
2. Reference ID: The common name for the SNP
3. Allele: The DNA base substitution cased by the SNP
4. Gene ID: The common name of the gene the SNP is located in

Appendix 4.6

Locus Name	Comment
ARK_rs41933917	SNP Failed
ARK_rs42026571	SNP Failed
ARK_rs42027576	SNP Failed
ARK_rs42223380	SNP Failed
ARK_rs42311145	SNP Failed
ARK_rs42580008	SNP Failed
ARK_rs43156472	SNP Failed
ARK_ss64726273_rs42819473	SNP Failed
ARK_ss95214853	SNP Failed
ARK_ss95214854	SNP Failed
ARK_ss95214856	SNP Failed
ARK_ss95214859	SNP Failed
ARK_ss95214861	SNP Failed
ARK_ss95214862	SNP Failed
ARK_rs29017177	SNP Uninformative
ARK_rs29024743	SNP Uninformative
ARK_rs41257079	SNP Uninformative
ARK_rs41666288	SNP Uninformative
ARK_rs41666290	SNP Uninformative
ARK_rs41699902	SNP Uninformative
ARK_rs41716552	SNP Uninformative
ARK_rs41716631	SNP Uninformative
ARK_rs41722114	SNP Uninformative
ARK_rs41746924	SNP Uninformative
ARK_rs41823113	SNP Uninformative
ARK_rs41840540	SNP Uninformative
ARK_rs41846165	SNP Uninformative
ARK_rs41861310	SNP Uninformative
ARK_rs41903246	SNP Uninformative
ARK_rs41909225	SNP Uninformative
ARK_rs41939754	SNP Uninformative
ARK_rs41976145	SNP Uninformative
ARK_rs42020089	SNP Uninformative
ARK_rs42021087	SNP Uninformative
ARK_rs42021387	SNP Uninformative
ARK_rs42021749	SNP Uninformative
ARK_rs42021755	SNP Uninformative
ARK_rs42022227	SNP Uninformative
ARK_rs42022743	SNP Uninformative
ARK_rs42023442	SNP Uninformative
ARK_rs42023448	SNP Uninformative
ARK_rs42023981	SNP Uninformative
ARK_rs42025129	SNP Uninformative
ARK_rs42025725	SNP Uninformative
ARK_rs42026557	SNP Uninformative
ARK_rs42027035	SNP Uninformative
ARK_rs42027059	SNP Uninformative

ARK_rs42027176	SNP Uninformative
ARK_rs42027230	SNP Uninformative
ARK_rs42027575	SNP Uninformative
ARK_rs42027632	SNP Uninformative
ARK_rs42027749	SNP Uninformative
ARK_rs42028278	SNP Uninformative
ARK_rs42028329	SNP Uninformative
ARK_rs42029155	SNP Uninformative
ARK_rs42030040	SNP Uninformative
ARK_rs42030070	SNP Uninformative
ARK_rs42032036	SNP Uninformative
ARK_rs42032370	SNP Uninformative
ARK_rs42032371	SNP Uninformative
ARK_rs42032378	SNP Uninformative
ARK_rs42034874	SNP Uninformative
ARK_rs42054102	SNP Uninformative
ARK_rs42109669	SNP Uninformative
ARK_rs42123134	SNP Uninformative
ARK_rs42189764	SNP Uninformative
ARK_rs42205048	SNP Uninformative
ARK_rs42239334	SNP Uninformative
ARK_rs42239339	SNP Uninformative
ARK_rs42254196	SNP Uninformative
ARK_rs42311136	SNP Uninformative
ARK_rs42312323	SNP Uninformative
ARK_rs42342086	SNP Uninformative
ARK_rs42345438	SNP Uninformative
ARK_rs42372315	SNP Uninformative
ARK_rs42447625	SNP Uninformative
ARK_rs42451592	SNP Uninformative
ARK_rs42451594	SNP Uninformative
ARK_rs42467700	SNP Uninformative
ARK_rs42468294	SNP Uninformative
ARK_rs42541025	SNP Uninformative
ARK_rs42557939	SNP Uninformative
ARK_rs42612805	SNP Uninformative
ARK_rs42612806	SNP Uninformative
ARK_rs42631147	SNP Uninformative
ARK_rs42745029	SNP Uninformative
ARK_rs42851411	SNP Uninformative
ARK_rs42851916	SNP Uninformative
ARK_rs43008782	SNP Uninformative
ARK_rs43054367	SNP Uninformative
ARK_rs43160956	SNP Uninformative
ARK_rs43281402	SNP Uninformative
ARK_rs43325273	SNP Uninformative
ARK_rs43346321	SNP Uninformative
ARK_rs43350855	SNP Uninformative
ARK_rs43434899	SNP Uninformative
ARK_rs43551637	SNP Uninformative

ARK_rs43556471	SNP Uninformative
ARK_rs43671260	SNP Uninformative
ARK_rs43689833	SNP Uninformative
ARK_rs43706486	SNP Uninformative
ARK_rs43706506	SNP Uninformative
ARK_rs43708798	SNP Uninformative
ARK_rs43715512	SNP Uninformative
ARK_rs43731750	SNP Uninformative
ARK_rs8193041	SNP Uninformative
ARK_rs8193050	SNP Uninformative
ARK_rs8193066	SNP Uninformative
ARK_rs8193069	SNP Uninformative
ARK_ss104796314	SNP Uninformative
ARK_ss104796336	SNP Uninformative
ARK_ss104796347	SNP Uninformative
ARK_ss28452040_rs17871699	SNP Uninformative
ARK_ss28452237_rs17871896	SNP Uninformative
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ARK_ss38336374_rs29023087	SNP Uninformative
ARK_ss46526981_rs41257426	SNP Uninformative
ARK_ss46527012_rs41255557	SNP Uninformative
ARK_ss62257493_rs41805428	SNP Uninformative
ARK_ss62359007_rs41848377	SNP Uninformative
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ARK_ss62453938_rs41882274	SNP Uninformative
ARK_ss62466877_rs41889047	SNP Uninformative
ARK_ss62466880_rs41889048	SNP Uninformative
ARK_ss62484289_rs41890230	SNP Uninformative
ARK_ss62485711_rs41894194	SNP Uninformative
ARK_ss62487088_rs41893053	SNP Uninformative
ARK_ss62490142_rs41893757	SNP Uninformative
ARK_ss62515930_rs41903052	SNP Uninformative
ARK_ss62518402_rs41899079	SNP Uninformative
ARK_ss62554507_rs41914650	SNP Uninformative
ARK_ss62619747_rs41931155	SNP Uninformative
ARK_ss62620131_rs41931981	SNP Uninformative
ARK_ss62625066_rs41939013	SNP Uninformative
ARK_ss62625201_rs41939858	SNP Uninformative
ARK_ss62626533_rs41935901	SNP Uninformative
ARK_ss62630137_rs41938903	SNP Uninformative
ARK_ss62778762_rs41994846	SNP Uninformative
ARK_ss62782137_rs41997469	SNP Uninformative
ARK_ss62782146_rs41997472	SNP Uninformative
ARK_ss62862759_rs42021824	SNP Uninformative
ARK_ss62865288_rs42022558	SNP Uninformative
ARK_ss62867832_rs42021406	SNP Uninformative
ARK_ss62877153_rs42023980	SNP Uninformative
ARK_ss62877252_rs42024011	SNP Uninformative
ARK_ss62928531_rs42041883	SNP Uninformative
ARK_ss62930439_rs42048416	SNP Uninformative

ARK_ss62935986_rs42045874	SNP Uninformative
ARK_ss62980002_rs42057017	SNP Uninformative
ARK_ss62980272_rs42057705	SNP Uninformative
ARK_ss62987289_rs42061802	SNP Uninformative
ARK_ss62988990_rs42058469	SNP Uninformative
ARK_ss62989689_rs42060100	SNP Uninformative
ARK_ss62997555_rs42074207	SNP Uninformative
ARK_ss63007716_rs42068083	SNP Uninformative
ARK_ss63528770_rs42263233	SNP Uninformative
ARK_ss63611805_rs42288095	SNP Uninformative
ARK_ss63648364_rs42300304	SNP Uninformative
ARK_ss63819411_rs42368097	SNP Uninformative
ARK_ss63888155_rs41811275	SNP Uninformative
ARK_ss63953452_rs42399876	SNP Uninformative
ARK_ss64036952_rs42441026	SNP Uninformative
ARK_ss64217528_rs42534616	SNP Uninformative
ARK_ss64314776_rs42573616	SNP Uninformative
ARK_ss64361987_rs42601449	SNP Uninformative
ARK_ss64487642_rs42661063	SNP Uninformative
ARK_ss64491234_rs42661323	SNP Uninformative
ARK_ss64578760_rs42710296	SNP Uninformative
ARK_ss64654695_rs42755290	SNP Uninformative
ARK_ss64722671_rs42820431	SNP Uninformative
ARK_ss64723613_rs42819260	SNP Uninformative
ARK_ss64751496_rs42852239	SNP Uninformative
ARK_ss64839202_rs42931384	SNP Uninformative
ARK_ss64960713_rs43049152	SNP Uninformative
ARK_ss65009674_rs43099540	SNP Uninformative
ARK_ss65056354_rs43144881	SNP Uninformative
ARK_ss65062768_rs43158855	SNP Uninformative
ARK_ss65201863_rs43284763	SNP Uninformative
ARK_ss65202748_rs43283828	SNP Uninformative
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ARK_ss65206126_rs43291704	SNP Uninformative
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ARK_ss65221173_rs43306573	SNP Uninformative
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ARK_ss65368172_rs43451327	SNP Uninformative
ARK_ss65370245_rs43457496	SNP Uninformative
ARK_ss65371577_rs43457274	SNP Uninformative
ARK_ss65371900_rs43455199	SNP Uninformative
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ARK_ss65466018_rs43551679	SNP Uninformative
ARK_ss65612945_rs43695847	SNP Uninformative
ARK_ss69357304_rs55617433	SNP Uninformative
ARK_ss69357307_rs55617183	SNP Uninformative
ARK_ss69357308_rs55617320	SNP Uninformative
ARK_ss69357334_rs55617136	SNP Uninformative

ARK_ss73689451_rs55617251	SNP Uninformative
ARK_ss73689452_rs55617270	SNP Uninformative
ARK_ss95214860	SNP Uninformative
ARK_ss95214864	SNP Uninformative
ARK_ss95214867	SNP Uninformative
ARK_ss95214874	SNP Uninformative
ARK_ss95214878	SNP Uninformative
ARK_rs17870216	SNP Fully Informative
ARK_rs17870353	SNP Fully Informative
ARK_rs17871880	SNP Fully Informative
ARK_rs41255556	SNP Fully Informative
ARK_rs41255662	SNP Fully Informative
ARK_rs41257015	SNP Fully Informative
ARK_rs41257112	SNP Fully Informative
ARK_rs41587590	SNP Fully Informative
ARK_rs41642092	SNP Fully Informative
ARK_rs41718866	SNP Fully Informative
ARK_rs41817317	SNP Fully Informative
ARK_rs41843359	SNP Fully Informative
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ARK_rs41915330	SNP Fully Informative
ARK_rs41932920	SNP Fully Informative
ARK_rs42027020	SNP Fully Informative
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ARK_rs42053876	SNP Fully Informative
ARK_rs42118884	SNP Fully Informative
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ARK_rs42309927	SNP Fully Informative
ARK_rs42312327	SNP Fully Informative
ARK_rs42342962	SNP Fully Informative
ARK_rs42502308	SNP Fully Informative
ARK_rs42578633	SNP Fully Informative
ARK_rs42688655	SNP Fully Informative
ARK_rs42932713	SNP Fully Informative
ARK_rs42978304	SNP Fully Informative
ARK_rs43396806	SNP Fully Informative
ARK_rs43397964	SNP Fully Informative
ARK_rs43596425	SNP Fully Informative
ARK_rs43703954	SNP Fully Informative
ARK_rs8193049	SNP Fully Informative
ARK_rs8193053	SNP Fully Informative
ARK_rs8193055	SNP Fully Informative
ARK_ss104796339	SNP Fully Informative
ARK_ss104796341	SNP Fully Informative
ARK_ss104796344	SNP Fully Informative
ARK_ss104796352	SNP Fully Informative
ARK_ss104796353	SNP Fully Informative
ARK_ss38325445_rs29011157	SNP Fully Informative
ARK_ss38326889_rs29015191	SNP Fully Informative
ARK_ss38330080_rs29021595	SNP Fully Informative

ARK_ss38337238_rs29026551	SNP Fully Informative
ARK_ss46526625_rs41257081	SNP Fully Informative
ARK_ss62004026_rs41726305	SNP Fully Informative
ARK_ss62488129_rs41895599	SNP Fully Informative
ARK_ss62488138_rs41895602	SNP Fully Informative
ARK_ss62491552_rs41897525	SNP Fully Informative
ARK_ss62492737_rs41890820	SNP Fully Informative
ARK_ss62530738_rs41910166	SNP Fully Informative
ARK_ss62611859_rs41935052	SNP Fully Informative
ARK_ss62625501_rs41940658	SNP Fully Informative
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ARK_ss62934126_rs42049356	SNP Fully Informative
ARK_ss63007899_rs42068744	SNP Fully Informative
ARK_ss63472982_rs42241895	SNP Fully Informative
ARK_ss64413920_rs42628233	SNP Fully Informative
ARK_ss64440170_rs42638947	SNP Fully Informative
ARK_ss64882909_rs42976134	SNP Fully Informative
ARK_ss64972149_rs43062765	SNP Fully Informative
ARK_ss65184322_rs43766721	SNP Fully Informative
ARK_ss65202157_rs43288056	SNP Fully Informative
ARK_ss65239654_rs43329536	SNP Fully Informative
ARK_ss65289507_rs43372164	SNP Fully Informative
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ARK_ss65415824_rs43494261	SNP Fully Informative
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ARK_ss65420027_rs43502712	SNP Fully Informative
ARK_ss65420029_rs43502714	SNP Fully Informative
ARK_ss65420031_rs43502716	SNP Fully Informative
ARK_ss65624825_rs43702384	SNP Fully Informative
ARK_ss69357306_rs55617309	SNP Fully Informative
ARK_ss69357318_rs55617354	SNP Fully Informative
ARK_ss69357319_rs55617174	SNP Fully Informative
ARK_ss69357322_rs55617351	SNP Fully Informative
ARK_ss69357324_rs55617390	SNP Fully Informative
ARK_ss69357336_rs55617258	SNP Fully Informative
ARK_ss73689408_rs55617385	SNP Fully Informative
ARK_ss73689445_rs55617168	SNP Fully Informative
ARK_ss73689467_rs55617437	SNP Fully Informative
ARK_ss73689478_rs55617324	SNP Fully Informative
ARK_ss73689479_rs55617311	SNP Fully Informative
ARK_ss73689485_rs55617297	SNP Fully Informative
ARK_ss95214857	SNP Fully Informative
ARK_ss95214858	SNP Fully Informative
ARK_ss95214863	SNP Fully Informative
ARK_ss95214865	SNP Fully Informative
ARK_ss95214869	SNP Fully Informative
ARK_ss95214873	SNP Fully Informative

ARK_ss95214875	SNP Fully Informative
ARK_rs17871955	Sample Clusters not as expected
ARK_rs29015593	Sample Clusters not as expected
ARK_rs29016829	Sample Clusters not as expected
ARK_rs41255407	Sample Clusters not as expected
ARK_rs41256961	Sample Clusters not as expected
ARK_rs41971213	Sample Clusters not as expected
ARK_rs42028267	Sample Clusters not as expected
ARK_rs42028513	Sample Clusters not as expected
ARK_rs42068459	Sample Clusters not as expected
ARK_rs42102914	Sample Clusters not as expected
ARK_rs42164128	Sample Clusters not as expected
ARK_rs42371911	Sample Clusters not as expected
ARK_rs42472240	Sample Clusters not as expected
ARK_rs42544474	Sample Clusters not as expected
ARK_rs42579977	Sample Clusters not as expected
ARK_rs42690103	Sample Clusters not as expected
ARK_rs42754503	Sample Clusters not as expected
ARK_rs43380660	Sample Clusters not as expected
ARK_rs43410172	Sample Clusters not as expected
ARK_rs43455095	Sample Clusters not as expected
ARK_rs43549594	Sample Clusters not as expected
ARK_rs43572154	Sample Clusters not as expected
ARK_rs43705168	Sample Clusters not as expected
ARK_rs43707857	Sample Clusters not as expected
ARK_rs43729240	Sample Clusters not as expected
ARK_rs55617204	Sample Clusters not as expected
ARK_rs55617272	Sample Clusters not as expected
ARK_rs8193048	Sample Clusters not as expected
ARK_ss104796338	Sample Clusters not as expected
ARK_ss28451414_rs17871073	Sample Clusters not as expected
ARK_ss62453668_rs41881585	Sample Clusters not as expected
ARK_ss62463331_rs41883772	Sample Clusters not as expected
ARK_ss62485393_rs41893490	Sample Clusters not as expected
ARK_ss62485639_rs41894171	Sample Clusters not as expected
ARK_ss62488132_rs41895600	Sample Clusters not as expected
ARK_ss62488144_rs41895604	Sample Clusters not as expected
ARK_ss62490139_rs41893756	Sample Clusters not as expected
ARK_ss62537242_rs41909114	Sample Clusters not as expected
ARK_ss62785710_rs42000457	Sample Clusters not as expected
ARK_ss62789211_rs41995921	Sample Clusters not as expected
ARK_ss62934615_rs42042620	Sample Clusters not as expected
ARK_ss63011817_rs42070647	Sample Clusters not as expected
ARK_ss63549708_rs42271298	Sample Clusters not as expected
ARK_ss64261740_rs42555252	Sample Clusters not as expected
ARK_ss64306304_rs42579196	Sample Clusters not as expected
ARK_ss64430642_rs42633118	Sample Clusters not as expected
ARK_ss64471540_rs42655015	Sample Clusters not as expected
ARK_ss64672574_rs42765470	Sample Clusters not as expected
ARK_ss64942091_rs43038502	Sample Clusters not as expected

ARK_ss65073178_rs43166805	Sample Clusters not as expected
ARK_ss65201725_rs43282727	Sample Clusters not as expected
ARK_ss65203854_rs43289334	Sample Clusters not as expected
ARK_ss65212328_rs43297955	Sample Clusters not as expected
ARK_ss65268174_rs43349824	Sample Clusters not as expected
ARK_ss65291092_rs43380234	Sample Clusters not as expected
ARK_ss65291390_rs43372439	Sample Clusters not as expected
ARK_ss65521671_rs43608009	Sample Clusters not as expected
ARK_ss73689448_rs55617330	Sample Clusters not as expected
ARK_ss73689455_rs55617166	Sample Clusters not as expected
ARK_ss73689477_rs55617197	Sample Clusters not as expected
ARK_ss73689504_rs55617254	Sample Clusters not as expected
ARK_ss95214855	Sample Clusters not as expected
ARK_ss95214868	Sample Clusters not as expected
ARK_rs41688025	Multiple Het clusters
ARK_rs42852439	Multiple Het clusters
ARK_rs43030763	Multiple Het clusters
ARK_rs43073975	Multiple Het clusters
ARK_ss104796340	Multiple Het clusters
ARK_ss104796354	Multiple Het clusters
ARK_ss62484286_rs41890229	Multiple Het clusters
ARK_ss62721129_rs41977113	Multiple Het clusters
ARK_ss63991193_rs42420659	Multiple Het clusters
ARK_ss65203499_rs43289279	Multiple Het clusters
ARK_ss65658944_rs41257282	Multiple Het clusters
ARK_ss73689415_rs55617317	Multiple Het clusters
ARK_ss73689416_rs55617267	Multiple Het clusters
ARK_rs42029541	Was unable to call discrete clusters
ARK_ss104796308	Was unable to call discrete clusters
ARK_ss104796350	Was unable to call discrete clusters
ARK_ss62067036_rs41743748	Was unable to call discrete clusters
ARK_ss65624885_rs43702941	Was unable to call discrete clusters
ARK_ss66538065_rs43708439	Was unable to call discrete clusters
ARK_ss69357321_rs55617145	Was unable to call discrete clusters
ARK_ss73689447_rs55617329	Was unable to call discrete clusters
ARK_ss73689469_rs55617137	Was unable to call discrete clusters
ARK_ss73689475_rs55617325	Was unable to call discrete clusters

Genotyped SNP information

This table contains the genotype information of each SNP. There are 6 classifications

1. SNP failed
2. SNP uninformative
3. SNP fully informative
4. Sample clusters not as expected
5. Multiple Het clusters
6. Unable to call discrete clusters