GENETIC ASSOCIATION OF TOLERANCE TO JOHNE'S DISEASE

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A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN ANIMAL SCIENCES

WASHINGTON STATE UNIVERSITY
Department of Animal Sciences

DECEMBER 2008

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of Ricardo Zanella find it satisfactory and recommended that it be accepted.

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Acknowledgments

I would like to thank Dr. Holly Neibergs, for accepting me to participate in her lab. Thanks for inspiring me, the excellent mentoring, and stimulating me to like science and genetics. A special thanks to Jan Vierck for always being around and ready to help me with all my difficulties. Thanks to Dr. Matt Settles, for his friendship and for the time that he spent in the lab teaching me bioinformatics, I would also like to thank my first advisor at WSU, Dr. Jerry Reeves. He was the person that brought me to WSU in 2006, and gave the opportunity to meet Dr. Neibergs. Thank you Dr. Reeves for all the time and advice that you gave me. I would like to thank Dr. Gaskins for always being available to give me good advice when I had questions about my project and for being a supportive member of my committee. To all the other professors in the Department of Animal Sciences and other Departments I would like to thank you for all the teaching.
To David, Nada and Jeanine a special thanks for the friendship and time that we have spent together.
For all my friends for showing me a wonderful time here in Pullman.
Most special thanks to my family, my father (Ipenor Zanella), my mother (Nair Zuleika Zandona Zanella), my sisters (Viviane and Valeria), my brother-in-laws (Flavio e Olando), and my nephew and nieces (Pedro, Laura, Paula and Helena). To my brothers (Adroaldo and Eraldo), sisters-in-law (Marcia and Janice), nephews (Mauricio and Lorenso), and nieces (Isadora, Giulia and Giovana), thanks for all the love, support, encouragement, and faith.

To Marina Lima, thanks for the help and for understanding me in this moment of my life.

Finally, thanks to all of those who have helped me.

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Abstract

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December 2008

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Johne's disease is a contagious disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map). This disease may potentially be zoonotic because of its association with Crohn's disease. To estimate the heritability of tolerance to Johne's disease, a definition of tolerance was determined by an index calculated by the average colony forming units (cfu)/gram of fecal and tissues values for Map at slaughter(average fecal culture + 100/average of tissue culture + 100). Ninety-four animals were tissue positive and used for the basis of the simulated data of 2500 offspring, 25 sires and 2500 dams. The estimated heritability of tolerance from the simulated data was $h^2 = 0.09 \pm 0.03$ using the MTDFREML program and Restricted Maximum Likelihood test. To identify the loci associated with tolerance, a whole genome association analysis was conducted with the Illumina Bovine SNP50 Bead array. Average tolerance and peak tolerance, using the highest fecal and tissue cfus/g for each animal instead of average value were used as phenotypes. After quality control filtering, 45,789 SNPs and 90 animals remained. The statistical analysis was conducted using the

R statistical environment and PLINK (version 1.04). There was no evidence of genetic variation between animals after evaluation of the genomic inflation factor, Q-Q or MDS plots. Strong evidence for association was identified with peak tolerance and a locus on BTA 15 ($p = 1.1\text{x}10^{-7}$, after Bonferroni correction $p = 0.005$), while moderate evidence for association ($p = 3.0\text{x}10^{-5}$) was identified on two adjacent SNPs on BTA 6. The same SNP on BTA 15 showed moderate evidence for association ($p = 2.1\text{x}10^{-6}$) with average tolerance. Two additional SNPs showed moderate evidence for association on BTA 2 ($p = 3.3\text{x}10^{-5}$) and BTA 1 ($p = 3.3\text{x}10^{-5}$). The estimation of the heritability of tolerance and the identification of loci associated with tolerance provides necessary information in the development of tools for selecting animals that are tolerant to Johne's disease. Selecting animals that are tolerant to Johne's disease may minimize animal and economic losses and reduce risks to human health.

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Dedication

This thesis is dedicated to the best guides that I have had in my life: my parents Ipenor and Zuleika. Thanks for all the stimulation, inspiration, support and teaching me that it doesn't matter how hard life is, we need to love it. No matter how cruel destiny can be, we need to support it and fight for our goals and then we will find a little bit of what is called HAPPINESS.

CHAPTER 1

LITERATURE REVIEW

1.1 BOVINE PARATUBERCULOSIS

1.1.1 Johne's Disease

Bovine paratuberculosis, also known as Johne's disesase, is a bacterial infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). All domestic and wild ruminants are susceptible to infection with Map, which is characterized by chronic enteritis. American bison, Tule elk, eastern whitetail deer, and feral rabbits have been implicated as potential carriers of Map that may influence incidence and spread of disease among wild and domestic animals [21,56]. Nonruminant animals can become infected experimentally; however, clinical disease usually does not develop [3,21,42,56,72,97].

The clinical symptoms, pathology and anatomical description of paratuberculosis were first described during the 19th century. The name Johne's disease comes from the work of H. A. Johne and L. Frothingham, who, in 1895, demonstrated a connection between cattle enteritis and the presence of acid-fast microorganisms in sections of the intestinal mucosa [24]. In 1906, Bang distinguished between tuberculosis and non-tuberculosis enteritis and proposed the name pseudotuberculosis enteritis. The identification of the etiological agent is attributed to F. W. Twort, who, in 1912, succeeded in cultivating and

characterizing a *Mycobacterium*, which in 1914 was shown to produce experimental enteritis in cattle [24]. After the full characterization of Map as a distinct species within the genus, the disease was renamed paratuberculosis [23,38,49]. Infection of Map early in life may lead to paratuberculosis after a long latency period that may last several years. Generally, the macroscopic and histological lesions are restricted to the intestines, associated lymph nodes and occasionally the liver [15,36]. Emaciation may be the only clinical sign in sheep and goats [64].

This disease has been recognized as a major animal health concern in many countries, including the United States, because it is incurable and is highly transmittable to other animals [22]. *Mycobacterium avium paratuberculosis* has been found in 25-75% of Crohn's disease patients, and in less than 5% of individuals without Crohn's disease suggesting a possible link between Crohn's disease and Map [1,7,20]. Both Johne's disease in cattle and Crohn's disease in humans are increasing worldwide in all industrialized countries [6]. It is not known if Crohn's disease is facilitated by Map or if Map is zoonotic to humans. However, most mycobacterial pathogens are vertically transmissible to humans and have the ability to cause disease. If Map is transmitted from cattle to humans, the most probable vehicle for this contamination is through milk and meat [6,19]. Conflicting studies have been reported as to the efficacy of milk pasteurization in killing the bacteria [84].

1.1.2 Economic Importance

On dairy farms, economic losses from Johne's disease occurs mostly through premature culling, reduced milk production, reproductive failure and weight loss in cattle. In a recent summary of production studies by Nordlund and colleagues,

Johne's-infected cows produced from 2 to 19% less milk then their uninfected herd mates [69]. According to Ott et al. Johne's positive herds generated $97 less annually ($1916 vs. $2013, $p < 0.01$) per cow when compared to herds without Johne's disease [71]. This was primarily due to a reduced average milk production of 288 kg (7515 kg vs. 7803 kg, $p < 0.01$) and increased costs of replacement cows.

For herds with greater than 10% of cull cows showing Johne's clinical signs, the economic loss was $245 ($1782 vs. $2027, $p < 0.01$) per cow. Similarly, Johnson-Ifearulundu and Kaneene reported that herds with Johne's disease produced 748kg less milk (7096 kg vs. 7844 kg, $p < 0.01$) or $214 less per cow [43]. Beaudeau et al. found a loss of 5.4 to 7.2 kg/day milk in cows with Map infection [10]. Nielsen and colleagues also showed a lowered milk yield in cows ($p < 0.05$) from herds containing at least one Map positive animal [67]. The decrease in milk production of an entire herd with only one animal with Johne's disease could be explained by the lack of sensitivity of the diagnostic tests [27,67]. Cows which were both ELISA positive and vaccinated had a smaller loss in milk yield per day than those that were unvaccinated. This may be due to a less severe disease in vaccinated animals or a cross-reaction from the vaccine resulting in a false-positive test [93].

Hendrick et al. showed that Map-infected cows had lowered milk production without an increase in somatic cell count (SCC) or differences in milk composition [37]. These results were in concordance with previous studies by Spangler et al. and Nordlund et al. in which they did not find differences in SSC between infected and non-infected cows [69,83]. Gonda and coworkers showed that infected cows with Map produced 303.9 kg less milk/lactation, 11.46 kg less fat/lactation, and 9.49 kg less protein/lactation ($p < 0.003$) [35]. Although infected cows had higher pregnancy rates (1.39%; $p = 0.0385$), they had lower

productive lives (2.85 months less; $p < 0.0001$).

1.1.3 Epidemiology

Paratuberculosis is a slowly spreading disease requiring an extended period of time for the development of clinical signs. By the time a single infected animal is identified, 38-42% of the herd may be infected [30,54,99].

1.1.4 Prevalence of the Disease

Despite efforts to decrease the prevalence of paratuberculosis, US dairy herds with infected animals have increased from 17% in 1990, to 22% in 1997 and finally to 67% in 2007 [5,13,68].

In a review paper, Lilenbaum and coworkers showed that approximately 17% of cattle in England presented with clinical paratuberculosis [57]. ELISA testing identified that 6% of dairy cattle in Belgium were seropositive for paratuberculosis. In the Netherlands, 2-6% of dairy herds were infected with Johne's disease with a prevalence of 31% to 71% within the herds [57]. A two-year epidemiologic study in Slovenia indicated a prevalence of paratuberculosis in 11% of national cattle [81]. Seventy per cent of herds in Denmark tested positive for paratuberculosis as determined by a serological study of bulk-tanked milk from 900 dairy herds [45]. Similar findings were described in Japan, Australia, New Zealand and other European countries [30,48,96]. In Buenos Aires, Argentina, where the disease was first detected in 1985, an epidemiological investigation demonstrated a that 26% of beef cattle and 56% of dairy cattle were seropositive [70]. In Brazil, the diagnoses of Johne's disease was first reported in imported animals, but now the disease has spread to native herds [77].

Efforts to reduce the prevalence of Johne's disease have had mixed results. The paratuberculosis control program proposed by the Australian government reduced seroprevalence to 1% and clinical cases to 0.1% over seven years [9]. However, the Johne's disease program in the United States has not resulted in a reduction in Johne's prevalence [5].

1.1.5 Clinical Signs

Paratuberculosis is a chronic, progressive granulomatous enteric disease of primarily ruminants. The clinical disease is characterized by diarrhea, weight loss, debilitation, and eventual death. Johne's disease typically exhibits a latent period after the infection and before the appearance of clinical signs, fecal shedding or antibody production. Annual death losses within a herd may be as high as 3 - l0%. Usually the clinical disease is associated with adult animals greater than two years-old; however, animals as young as 4 months old may occasionally develop clinical signs [80].

Cattle that develop clinical signs of Johne's disease have thickened intestinal mucosa, resulting in diarrhea that is not responsive to treatment and decreased intestinal absorption of nutrients. Edemas of the throat and abdomen, loss of coat color and emaciation have also been observed [23,75].

1.1.6 Transmission

The infection of calves can take place by oral ingestion of the bacterium from infected manure, colostrum or milk [85]. It is generally assumed that animals start shedding the bacteria in the feces at approximately two years of age and do not transmit the disease before that age [23,89]. However, fecal shedding of the

bacterium may occur in the first few weeks after oral inoculation with high infectious doses.

The presence of fecal shedding of Map may not always be indicative of an animal with Johne's disease. In herds with high environmental levels of Map, ingestion of Map may result in the presence of the bacterium in the feces even though the animal does not become infected. This has been characterized as pass-through of Map or a pass-through animal [10]. It is not understood why some animals remain uninfected after ingestion of Map while others succumb to the disease. The presence of pass-through animals complicates the interpretation of the definition of an animal with Johne's disease by fecal diagnostic testing.

Fetuses

Alexejeff-Goloff published the first report of bovine fetal infection with Map [8,66]. Infection of the fetus may occur after day 60 of gestation and may involve a wide range of fetal organs, fetal membranes and the structural elements of the placenta [51]. In ewes with clinical disease, 83% of their fetuses had evidence of Map infection whereas only 2% of ewes with sub-clinical disease had fetuses with evidence of Johne's disease [74]. Fetuses from cows with clinical disease are 12.5 times more likely to be infected with Map than fetuses from cows that have sub-clinical infections [96].

Calves

Calves are highly susceptible to Johne's disease. The major route of transmission is from an infected dam to her offspring through contaminated fecal material on the teats or contaminated colostrum and milk [23]. Other means of transmission include direct contact of calves with infected animals, contaminated pasture, feed

and water. Infected animals have a common fecal shedding pattern. Shortly after Map intake, fecal shedding of Map is detected for a few weeks, after which it declines to an undetectable level. This marks the beginning of the latency period where fecal shedding may be absent for several months to years. Near the onset of clinical signs, the presence of Map will again be detectable in the feces.

Cows

Johne's infection in adults is not well understood. Some animals exposed for the first time as adults may develop clinical disease, but this has not been experimentally proven. It is hypothesized that animal's that become infected as adults may become carriers of the organism without manifesting clinical signs just as animals that are infected as calves [53].

Latency Period

Most of the infection with Map occurs at young ages, but the calves do not generally manifest clinical signs until adulthood. This delay from the time of infection until the presence of clinical signs is called the latency period which may range from 2 to 10 years [9,84,94]. This period was well described by Whitlock and Buergelt and consists of four different stages [95]:

1. Latent stage: This stage represents young animals up to two years of age that do not show clinical signs of the disease. At this early stage of infection, animals shed the organism in undetectable levels. Detection of Map during this stage is only possible by culture of tissues.

2. Sub-clinical stage: This stage typically occurs in adult animals without visible clinical signs of Johne's disease. At this stage, antibodies and cell-mediated

immune responses against Map may be detected. Only 15-25% of cases of infected animals are detectable by fecal culture at this stage.

3. Clinical paratuberculosis: Clinical paratuberculosis results after Map levels have risen and clinical symptoms such as intermittent diarrhea and weight loss begin to appear. Clinical disease occurs in cattle most of the time from two to six years after the infection. Some animals may recover to the second stage, while the majority progress to the fourth stage with persistent diarrhea. Fecal culture and ELISA testing of these animals are positive.

4. Advanced stage of clinical paratuberculosis: Edema of the throat, cachexia and persistent diarrhea are characteristics of this stage. Most of these animals are culled or die of dehydration and cachexia.

As alternative to Whitlock and Buergelt description of Johne's disease, Cocito and Tiwari suggested that paratuberculosis in cattle progresses through three distinct stages, where in stage two animals may present some general signs of disease that are not specific to Johne's, while stage three and four are combined into one stage [24,87].

1.1.7 Diagnostic Tests

Low sensitivity of diagnostic tests is often cited as a major problem in the control of bovine paratuberculosis. The available tests for Johne's disease are not 100% sensitive, resulting in an inability to identify all the infected animals and resulting in false-negative results. Some tests produce substantial numbers of false positives, contributing to the appearance of an increased prevalence of paratuberculosis [23].

Fecal culture and serological testing are dependent on the stage of the disease, having higher sensitivity in the late stages [44,55,63]. Collins et al. showed that

the examination of multiple tissues at necropsy is the most sensitive diagnostic test to identify infected animals with paratuberculosis [26].

ELISA- Enzyme-Linked ImmunoSorbent Assay

Tests for the detection of antibodies to Map, such as ELISAs, have a moderate-to-low sensitivity because of the variability of the animal's immune response with the stage of the disease. The ELISA test rarely gives a positive result in animals under two years of age and frequently fails to detect individuals in the early phases of infection [46]. There are multiple commercial ELISA tests available for Map. In North America, there are three USDA approved ELISAs: ELISA A (Herdchek ELISA, IDEXX Laboratories, Westbrook, Maine), ELISA B (Parachek ELISA Biocor Animal Health, Omaha Nebraska, currently Pfizer Animal Health) and ELISA C (Synbiotics, San Diego, CA, USA). McKenna compared the three ELISA tests and found low agreement between the different commercially available ELISA kits, as a result of the low sensitivity of these tests [61].

Studies indicate that vaccinated herds have a higher percentage of ELISA-positive cattle probably due to the cross reaction of the antibodies made against the vaccine [65]. Vaccination of cattle against Johne's disease reduces the severity of disease in infected cattle but does not reduce the incidence of disease [50, 93].

Sensitivity and Specificity

Sensitivity is defined as the proportion of true positives of all the disease cases in the population. Specificity is the proportion of true negatives of all negative cases in the population.

The sensitivity of the ELISA has been estimated using fecal culture as the gold standard. The ELISA sensitivity was high (87%) for clinically affected, high shedding cattle and was low (15%) for sub-clinically infected, low shedding cattle [86].

$$Specificity = \frac{True\ Negatives}{True\ Negatives + True\ Positives} \tag{1.1}$$

Whitlock and colleagues showed that the specificity of the ELISA is high (98.9%) [94]. However false-positive test results are possible and a suitable confirmation test would be desirable to estimate what percentage of the ELISA-positive cattle are fecal culture-positive. Collins and coworkers evaluated five different ELISA tests and found that the specificity among them was approximately 84.7% (95% CI: 82.9 to 86.5%) [27].

Fecal Culture

The sensitivity for fecal culture is directly related to the stage of infection. Stage 1 (sub-clinical, un-detectable shedding of Map) represents 62-70% of all infected animals [25,94]. In stage 2, more animals will be shedding the microorganism and will be detected by fecal culture, although they do not yet show clinical signs of disease. In stage 3, animals will have clinical signs of the disease and will be detectable by fecal culture.

Sensitivity and Specificity

Whitlock et al. found that the sensitivity of detecting a positive animal with just one fecal sample was 38% [94]. If repeated fecal samples were taken at 6 month

intervals, sensitivity increased to 42%. Although the sensitivity of fecal testing is lacking, the specificity of fecal culture is reported to be 100% [15,23,94].

Culture of Tissues for Map

Disseminated infection is diagnosed when Map is isolated in tissues other than the intestines or their associated lymph nodes and is distinguished from infection found only in the gastrointestinal tissues and from absence of infection [4]. The primary tissues infected with Map are those related to the gastrointestinal tract such as the intestines and mesenteric lymph nodes [36]. Once infection occurs, Map spreads to other tissues such as the liver, kidney and spleen [95].

Sensitivity and Specificity

The isolation and culture of Map from tissue is considered to be the most precise test to identify and confirm animals with Johne's disease. According to the level and time of infection, animals will present a high number of colony-forming units (cfus/gram(g)) ranging from 1 to millions of cfu/g of tissue. Unfortunately, culture of tissue is not available on a commercial basis, is time consuming and is prohibitively expensive.

1.2 TOLERANCE

1.2.1 Infection

Infection is the colonization of the host by a foreign pathogenic or non-pathogenic species. Generally the infectious organism utilizes the host resources to proliferate. Pathogenic microorganisms can cause health problems in animals, and if left untreated, may result in death. Animals that have the capacity to

prevent infection or growth of the pathogen are known as resistant. Animals that are able to reduce the effect of the pathogen or lessen its severity are referred to as tolerant [14,73].

1.2.2 Definition of Tolerance

Tolerance is one mechanism of response to infection by pathogenic microorganisms. It is characterized by the ability to limit the disease severity induced by a pathogen [33,73]. It is not known whether animals show genetic variation for tolerance but there is evidence suggesting tolerance exists in mammals.

Alpha thalassemia is a heritable hemoglobinopathy caused by the reduced synthesis of α-globin chains that form part of normal adult hemoglobin (Hb). There are two forms of the α thalassemia gene in the human population [32]. Inheritance of two mutant forms (-/-) results in an inherited autosomal recessive blood disease that causes anemia. In regions where malaria is a persistent problem, an increase in the proportion of wild type heterozygotes (-/+) and mutant homozygotes (-/-) are found. This is due to the protective effect of this mutation in reducing the severity of malaria infection with *Plasmodium falciparum*, although it does not reduce the infection of the parasite. Thus, it seems that α-thalassemia affects tolerance (ability to limit the severity of the disease) but not resistance (ability to limit the infection) to *Plasmodium falciparum* [34,73,91].

It is hypothesized that cattle may exhibit genetic tolerance to Johne's disease. Genetic tolerance to Johne's disease is defined by the level of fecal shedding compared to the level of tissue infection of cows [99]. A tolerant animal would have the capacity to reduce the spread of the microorganism through the feces, limiting the transmission of Map to the herd, thereby reducing the incidence of

new cases. Fecal shedding was chosen as a measure of tolerance as it is correlated with disease severity, decreased milk production, fat content, protein content, lower productive life and increased number of days open [11,35,43,62,69,98]. The use of fecal shedding provides the ability to quantify the severity of the disease and to compare the severity of the disease with the level of infection in the tissues.

1.2.3 Methods to Measure Tolerance

Genetic tolerance to disease has currently only been proven in plants. In infected plants, tolerance is measured as the severity of the disease at a given level of infection. A tolerant plant is one that has a parasite infection but does not show signs of the disease (reduced disease severity). Plant disease severity is measured by the detrimental effects of the parasite, such as decreased production of flowers, grain, biomass, and nutritional composition of the plant [14,17].

In animals, we suggest that tolerance be measured by alterations of the physiological functions of the host. Physiologic changes reflect the severity of the disease and may be captured through weight loss, behavior changes, loss of production, infertility, and illness [73].

1.2.4 Importance of Tolerance

Genetic selection for health traits has previously been based on identifying animals that are susceptible or resistant to disease. Selection of animals for genetic tolerance would be based on animals that could limit the severity of the disease. Therefore, tolerant animals may or may not be resistant to disease, and resistant animals may not be tolerant. Selection of animals for tolerance places different evolutionary pressures on disease pathogens and the host than selection for resistance.

For some diseases, selection for tolerance and resistance may provide the best strategy for improving animal health, whereas in other diseases this may not be the case. The ability to select animals based on genetic tolerance and/or resistance can be used as a new approach to select animals that respond to specific pathogens. In diseases that are not transmitted by fecal shedding, the reduction in tolerance may have little effect on disease incidence but may have a major impact on the economic losses sustained due to lowered production.

1.3 HERITABILITY

1.3.1 Definition of Heritability

In general terms, narrow-sense heritability represents the proportion of additive genetic variance between individuals in a population. Heritability is a quantitative estimation of variation to a specific trait that is passed from parents to offspring. Narrow-sense heritability (h^2) is calculated by the correlation (r^2) between the phenotypic variation (P) and breeding value (BV) or $h^2 = r^2_{P,BV}$ [40].

Breeding value is defined as the value of the genes of the parent that may be transmitted to the progeny and is related to the additive effect. A phenotype is an observed trait that is associated with animal performance, health or physical appearance. Estimation of heritability ranges from 0 to 1. Values closer to zero (0) reflect that the trait has a lower heritability, while values close to one (1.0) describe a trait with a higher heritability. If a specific trait has a high heritability ($h^2 \geq 0.30$) and population variation exists, rapid gains in genetic improvement may be made. In this case, animals with high phenotypic performance would tend to produce offspring with high phenotypic performance. However, if a trait

has a low heritability, performance records from the parents will not be good estimates of the performance of the offspring.

1.3.2 Uses and Limitation of Heritability

Heritability and phenotypic variance are prediction terms used to estimate expected gains from selection for a specific trait. The estimation of these variances are especially important in the improvement of polygenic traits [12]. Some traits have low heritability, where the environmental effects are the most important determinants of success. When traits have lower heritabilities, the prediction of breeding values are less accurate, which can lead to selection errors and slower genetic progress [12].

It is important to remember that an estimate of heritability is within the context of a specific population of animals in a given environment. The heritability of a trait is not fixed: it varies from population to population and environment to environment. Because of the effect of the environment and the differences in genetic variation between groups of animals, data from animals of different origins should be analyzed by contemporary groups. Contemporary groups are groups of animals that have experienced a similar management, environment, location, and genetic background [40].

1.3.3 Methods to Estimate Heritability

Animal Model

The animal model is a mixed model, described by linear regression that refers to a system of dam and sire evaluations. In this model the investigator chooses the number of generations to be evaluated of the animals under study. The

relationships between animals in the extended pedigree as well as the animal's own performance will be used to calculate the heritability [59]. The animal model is expected to provide estimates of the transmitting ability with higher precision than estimates restricted to the similarity between close relatives [52, 58]. Moreover, the animal model is expected to be statistically more robust to unbalanced data sets compared to parent-offspring models [2].

The animal model contains a mixture of fixed and random effects. Fixed effects are unknown constants that affect the mean of a distribution. Random effects describe factors with multiple levels sampled from a population. Loeske and Kruuk suggested that random effects influence the variance of the trait and therefore the vaciances are considered to be additive [58]. This model assumes a random animal genetic effect for a phenotype Y of individual *i*.

$$Y_i = \mu + a_i + e_i \tag{1.2}$$

Where μ is the population mean, a_i is the additive genetic of individual *i*, and e_i is a residual error [59]. A more complex animal model was described by Akesson and colleagues [2]:

$$Y = X\beta + ZA_a + ZC_c + ZM_m + ZN_n + e \tag{1.3}$$

Where y is the vector of observed phenotypic values of the individuals and vectors, β = fixed effects, *a*=additive effects, *c*= permanent environment effects, *m*= maternal effects, *n*= common-nest effects and *e*= residual effects. X, ZA, ZC, ZM and ZN are design matrices relating the records to the appropriate fixed and random effects [59]. The *c, m, n* and *e* are refered as environmental effects and their variance as environmental variance.

Inbreeding Coefficient (Fx)

The inbreeding coefficient *(Fx)* measures the level of inbreeding in an individual. It is the probability that both genes of a pair are identical by descent [88]. Two genes are identical by descent if they are exact copies of a single gene inherited from a common ancestor in both parents of the animal.

The inbreeding coefficient is a probability, and it ranges from 0 (outbred) to 1 (highly inbred) [12]. The level of inbreeding of an animal can be determined by the degree of relatedness of its parents.

$$F_x = \sum 1/2^{n_1} +^{n_2} +_1(1 + F_A) \tag{1.4}$$

Where F_x is the inbreeding coefficient of the animal, F_A is the inbreeding coefficient of the common ancestor, n_1 is the number of generations from the sire to the common ancestor, and n_2 is the number of generations from the dam to the common ancestor [12, 82].

1.4 ASSOCIATION STUDIES

1.4.1 Genotyping and Quality Assessment

Whole Genome Association Analylsis

Whole genome association analysis is a powerful method to identify loci associated with complex diseases. Case control studies have been primarily used to identify these loci [39]. Whole genome association analysis for cattle is possible through the use of the new Illumina BovineSNP50 BeadChip. This genotyping resource provides genotypes of more than 50,000 single nucleotide polymorphisms (SNPs) across the bovine genome [29, 78]. This technology can be

used to identify loci associated with disease [90]. Marker assisted selection holds promise for use in the cattle industry to select genetically superior animals for traits associated with diseases, or select for traits that are expensive or impossible to measure in conventional breeding programs [28,47,78,90].

Data Quality Assessment

Genotyping Call Rate

The overall call rate of a sample is equal to the number of SNPs receiving an AA, AB, or BB genotype divided by the total number of SNPs. If the overall call rate for a SNP is less than 90% (no call rate $>$ 10% across the entire sample set) it is considered to be a poor performer and is subsequently removed from the analysis [28,41,78]. Degraded DNA samples, polymorphisms surrounding the interrogated SNP, or poor assay design may result in lower call rates [28,41].

Hardy-Weinberg Equilibrium

Hardy-Weinberg (HW) equilibrium states that genotype frequencies in a population should remain constant or are in equilibrium from generation to generation ($p^2 + 2pq + q^2$) unless specific disturbing influences are introduced. Because HW is a robust measure across the genome, HW may be used to identify regions of genotyping error by identifying SNPs that are not in HW equilibrium. Violations of the HW assumptions could also explain deviations from HW. The assumptions of HW are infinite population size, random mating, lack of inbreeding, assortative mating, selection, mutation and migration.

Non-Informative Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) were identified for this assay by sequencing of DNA from Holsteins and comparing it with DNA sequence obtained from other breeds as well as by a novel identification method [90]. Within any given breed of cattle there exists the possibility that some SNPs will be monomorphic. For association analyses, only heterozygous SNP's are informative and will be utilized.

Unassigned SNPs

The majority (97%) of SNPs that comprise the Illumina BovineSNP50 Beadchip assay are assigned to the bovine physical map through sequence data or other methods. SNPs that are unassigned are not useful in identifying chromosomal regions that harbor loci associated with a trait. Unassigned SNPs will be removed from the association analylsis as they do not provide information about the location of associated loci.

1.4.2 Loci Associated with Diseases

Association studies use analytical methods to identify loci that are associated with a trait. According to Risch and Merikangas, the power of association studies is greater than the power of linkage studies for the identification of complex traits [76]. Genetic association studies correlate differences in disease frequencies between groups with differences in allele frequencies at a given locus. Association analyses may utilize population-based or family-based tests. Population-based tests compare differences in allele frequencies between unrelated animals with and without a given trait. In this type of analysis, it is important to control for

population stratification to avoid spurious false positives. A spurious association may occur when a case and control have different allele frequencies due to an underlying genetic diversity that is unrelated to the trait or is due to population stratification [16].

Cavalli-Sforza et al. reported that variation exists between allele frequencies in different populations, because each population has a unique genetic history [18]. Thus, ancestral patterns of geographical migration, mating practices, reproductive expansions and bottlenecks may result in allele frequency differences between animals that are not related to the trait under study [16,79]. To control for possible population stratification, unrelated animals may be matched for potential confounding factors such as age, gender, environment and breed. Population stratification could result in increased type I error or false positive result [60].

New association analysis of human studies using unrelated individuals has demonstrated that population stratification is not as much of a problem as was once feared [92]. Several statistical tools can be used to detect and adjust for stratification [60]. Another approach to limit population stratification is to use familybased tests. The most common family-based test used is the Transmission Disequilibrium Test (TDT). This method tests for equality in transmission and non-transmission of a given allele to affected offspring from heterozygous parents [31]. This test requires that an affected animal have heterozygous parents. An advantage of the TDT is that genetic variation due to geographical migration, mating practices, reproductive expansions and bottlenecks, and stochastic variation will be similar in all family members [31]. A disadvantage of the TDT is the loss of power that results from using family data.

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CHAPTER 2

HERITABILITY OF TOLERANCE TO JOHNE'S DISEASE IN CATTLE

2.1 ABSTRACT

Johne's disease, also known as bovine paratuberculosis, is a highly contagious bacterial disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Once infected with Map, animals may remain asymptomatic for several years prior to the appearance of clinical signs and detection by ELISA or fecal culture. Prevention of Johne's disease by vaccination or treatment of the disease by antibiotics has not proven successful. Consequently, the prevalence and economic loss associated with Johne's disease in the United States continues to rise. The objective of this study was to estimate the heritability of tolerance to Johne's disease, as selection of cattle that are tolerant to Map may offer a potential mechanism for limiting the impact of this disease in cattle. Two hundred and forty five animals from four dairy herds were tested for Map by fecal culture and culture of tissue at the time of slaughter. Tolerance (the average fecal colony forming units (cfu_f)/gram(g) of 4 fecal replicates divided by the average tissue count in colony forming units of 4 tissues, each with 4 replicates (cfu_t)/gram(g)) was calculated for 94 animals whose tissues demonstrated evidence of Map. A simulation of 5025 animals was then conducted based on these tolerance values. The simulation included 25 sires, each mated to 100 dams, to produce 2500

offspring. An animal model and MTDFREML software were used to estimate the heritability. The estimated heritability of tolerance to Johne's disease was 0.09 $\pm$ 0.036. These data suggest that selection of cattle tolerant to Johne's disease may provide an alternative means of reducing the impact of this disease.

2.2 INTRODUCTION

Johne's disease is a prevalent and economically important disease of cattle, caused by bacterial infection with Map [5,17]. Transmission of Map is most common through ingestion of contaminated fecal material in calves but infection in adult cows has been observed. Transmission may also occur through semen, milk or across the uterus to infect the fetus. Johne's disease often results in lowered milk production, intermittent diarrhea, weight loss and death [13,19,20,24].

Current diagnostic methods detect less than 25% of infected animals at all stages of disease [6,8]. By the time a single infected animal is identified, it is estimated that 38-42% of the herd is likely to be infected [9]. The disease prevalence in US dairy herds has increased over 300% in the past 17 years, increasing from 22% in 1997 to 67% in 2007 [1].

Eradication of the disease has not been successfully achieved by traditional methods, and new approaches are needed. One approach is the selection of animals that are tolerant to Johne's disease. Cattle have evolved defense strategies such as resistance and tolerance to fight pathogens. Co-evolution between hosts and pathogens is believed to have generated substantial biological diversity [3].

The evolutionary consequences for the host and the pathogen will differ for defensive strategies utilizing resistance compared to tolerance. Tolerance reduces the harm that infection causes the host, whereas, resistance reduces the number of animals that will become infected. For the pathogen, tolerance results in a

reduction of virulence, thereby increasing selection pressure for pathogens to exhibit higher growth rates. For the host, tolerance will tend to increase disease prevalence, while reducing the individual risk of death from the disease [18].

Selection for resistance puts increasing pressure for virulence on the pathogen and for fitness in the host. Resistance to pathogen strains is frequently highly specific and may result in different costs to the hosts depending on whether the resistance is expressed early or late in life [3,4]. The interaction of the defense mechanisms of tolerance and resistance may result in the decline of one mechanism in favor of the other. This selection has been postulated to be one source of maintenance of genetic variation in both traits [14].

The optimal balance of tolerance and resistance would be expected to be unique for each host and pathogen and for the amount of genetic diversity in the host and pathogen populations [11]. Previous studies estimating the heritability of Johne's disease in cattle have focused solely on the defense mechanism of resistance. The objective of this study is to estimate the heritability of tolerance of Johne's disease.

2.3 MATERIALS and METHODS

2.3.1 Calculation of Tolerance

Four Holstein dairies were observed for four or more years. When animals were culled for any reason, tissue was obtained from the ileum, ileocecal valve and two ileocecal lymph nodes. Culture of the tissues and feces was conducted as described previously [27,28]. Tissue sampling indicated that 41% of herd A, 15% of herd B, 45% of herd C and 69% of herd D were infected with Map. Fecal sampling was completed every 6 months for cows in herd A, B, and C, and every

4 months for cows in herd D. The percentage of positive fecal samples in the last year of testing ranged from 1.5-5.2%. A total of 245 animals were culled with 94 animals demonstrating evidence of Map in their tissues. These cattle served as the source for the simulated data of 5025 animals to determine the heritability of tolerance of Johne's disease.

To calculate the tolerance average, the average level of Map in colony forming units per gram of tissue for 4 replicates from the 4 tissues was determined. The average colony forming units (cfus)/gram(g) of feces for each animal was also compiled at slaughter. Four replicates of each fecal specimen were used to determine the average fecal culture value for each animal. Tolerance was then determined using **Equation 2.1**.

$$AverageTolerance = \frac{cfu_f + 100}{cfu_t + 100} \tag{2.1}$$

The value of 100 was added to the cfu values so that animals with cfu$_f = 0$ would generate a tolerance value. Animals that had a tolerance index value greater than 1 were considered intolerant because the cfus/g from fecal culture exceeded the cfus/g from the culture of tissue.

2.3.2 Simulation

Tolerance values were simulated for 25 sires each mated to 100 dams and their offspring. Data from the 5025 simulated animals was based on mean and variance tolerance values measured from infected animals from four different dairy herds.

Simulation of 25 sires were increased to 100 sires to determine if sire breeding values (bvs) would be more normally distributed. The simulation was done using the R statistical environment [26]

$$y_i = \mu_t + 0.5(bvs) + 0.5(bvd) + \sqrt{0.5} * N(0, \sigma_a^2) + N(0, \sigma_e^2) \quad (2.2)$$

Where:

- y_i= tolerance for individual offspring *i*
- μ_t= tolerance mean
- *bvs*= sire breeding value, calculated by a pseudo normal number $(0, sd_g)$,with mean equal zero and the standard deviation obtained from values across herds (global values).
- *bvd*= dam breeding value, estimated by a pseudo normal number $(0, sd_{cg})$, where dam tolerance was estimated using data from their respective herds(CG)(sd_{cg}) and/or global values.
- $\sqrt{0.5}$ x pseudo normal number $(0, \sigma_a^2)$ = Mendelian genetic segregation and recombination.
- σ_a^2 = 10% of the standard deviation across herds(sdg) $\sigma_a^2 = 0.1$, $(0.345)2 = (0.11)$.
- $rnorm(0, \sigma_e^2)$ = environment effect (90% of the standard deviation across herds (sdg) $se^2 = (0.345)^2 - 0.1(0.345)^2$)

2.3.3 Heritability Estimation

An animal model was used to estimate the heritability for tolerance to Johne's disease using the MTDFREML program [2]. The simulation created a pedigree file

composed of sire, dam, offspring and the animal's own phenotype (tolerance). Heritability of tolerance to Johne's disease was estimated using the model:

$$y_{ij} = [\mu + CG_i] + G_{ij} + e_{ij} \tag{2.3}$$

Where:

- y_{ij}= tolerance value of *j*th animal in the *i*th contemporary group
- μ = overall tolerance mean (1.21)
- CG_i = effect of ith herd effect (fixed effect)
- G_{ij} = additive genetic effect of *j*th animal in the *i*th herd
- e_{ij} = residual of *j*th animal in the *i*th herd

The starting variance for additive effects was 0.076. This value was 67% of the global simulated tolerance variance across the herds. The residual comprised the remaining 33% of the variance. The program was run until convergence to obtain phenotypic variance and covariance estimates. Heritability was then calculated using $h^2 = {\sigma_a}^2/{\sigma_p}^2$ where ${\sigma_a}^2$ is the additive genetic variance and ${\sigma_p}^2$ is the phenotypic variance [2,29]. In this model, contemporary group (herd) was treated as a fixed effect, because different environmental factors and management techniques after calving have been associated with increased incidence and prevalence of Johne's disease [7,10,25].

2.4 RESULTS and DISCUSSION

The distribution of tolerance values for Johne's disease of the simulated offspring is shown in **Figure, 2.1**.

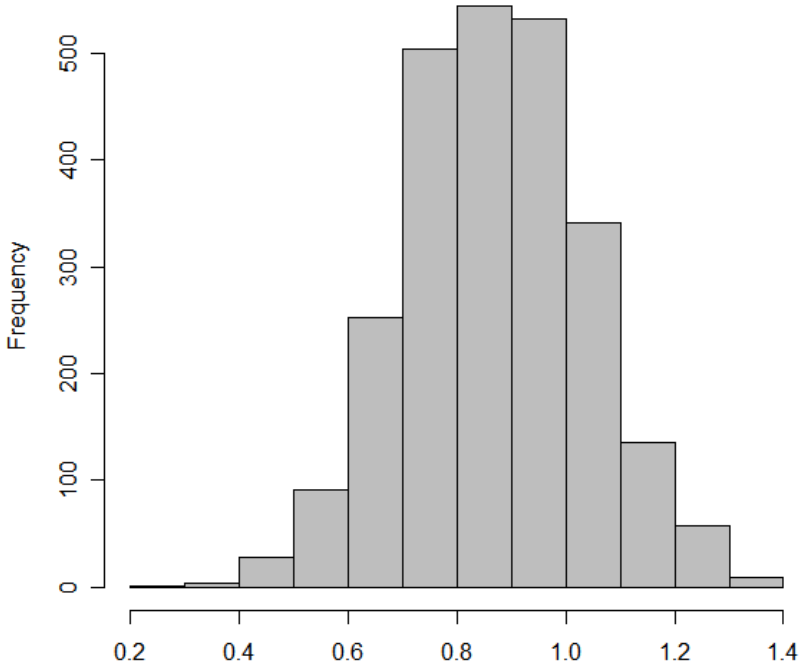

Figure 2.1: The distribution of the tolerance values of Johne's disease in the simulated offspring. Values ≥ 1 represent animals that are intolerant. Values < 1 represent animals that are tolerant.

Increasing the number of sires from 25 to 100 did affect the distribution of sire breeding values, resulting in a normal distribution, however it did not affect the the mean variance or the estimate of the heritability **Figure 2.2**.

The narrow sense heritability (h^2) of genetic tolerance to Johne's disease in dairy animals using a contemporary group as a fixed effect was estimated to be 0.09 ± 0.036. When contemporary group effect (herd) was used as a random effect, there was no effect on heritability values for tolerance to Johne's disease ($h^2 = 0.08 \pm 0.033$). This heritability estimate (0.09) is comparable to heritability estimates of resistance or susceptibility to other diseases [12,16,21,22].

Simianer and colleagues estimated that the heritability for disease traits in Norwegian cattle was 0.05 to 0.13 [22] . A Dutch study of postmortem tissues reported that the heritability for susceptibility to Johne's disease was 0.09 in a population of vaccinated animals, 0.01 in non-vaccinated animals, and 0.06 in an overall population [16]. Gonda and colleagues used a combination of ELISA and fecal culture to estimate the heritability of resistance to Johne's disease at 0.159 [12]. Similar findings were found in German studies conducted by Hinger et al. demonstrating a heritability of 0.05 to 0.13 for antibody response to Map in dairy cows [15].

In the current study, many of the animals with evidence of Map in their tissues were at low levels ($\leq$ 25 cfus/g of tissue). These animals typically were not fecal positive, which limited the information available for the determination of tolerance. Animals with tissue cfus/g $\geq$ 100 were often fecal positive. These cows had high levels of Map in their tissues and the highest levels of fecal Map shedding. Few of the animals in this study had high levels of Map infection.

Animals highly infected with Map may provide more information in regard to animals that are intolerant to Johne's disease. Animals with lower levels of tissue

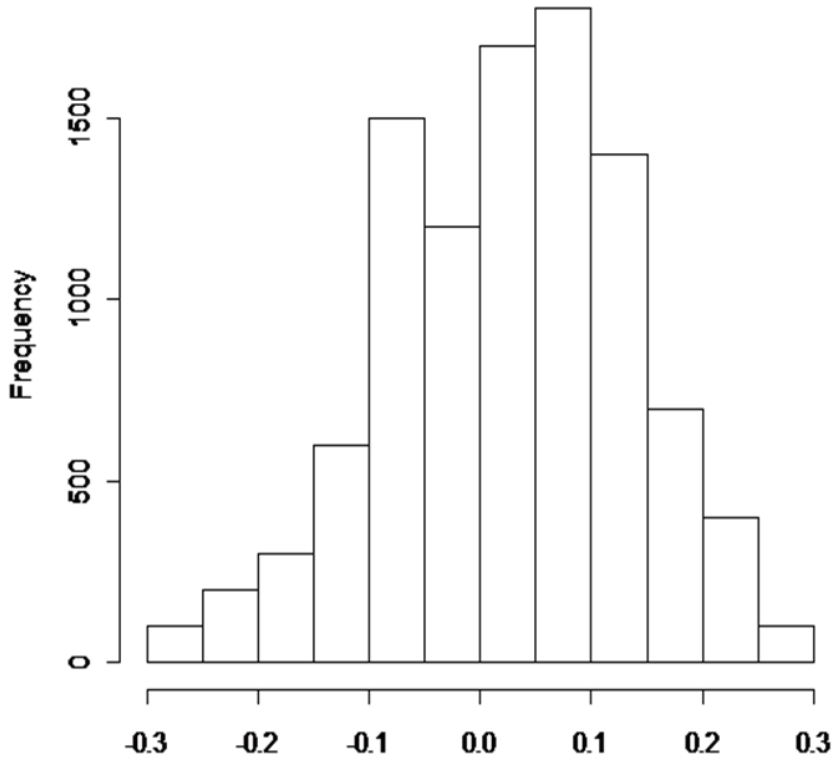

Figure 2.2: Distribution of Sire Breeding Value for tolerance, using 100 sires producing a normal distribution.

infection and low or no fecal sheding tend to be tolerant to Johne's disease. This study defines tolerance as the ability of the animal's genotype to sustain a particular level of tissue damage with relatively less bacteria present in the feces than other genotypes experiencing the same level of damage.

Tolerance is an alternative to resistance as an evolutionary response to selection imposed by pathogens [23]. Tolerance is expressed only if an animal experiences damage (in this case tissue damage) in animals with low levels of resistance. Genotypes with high tolerance will experience little selection for resistance just as highly resistant genotypes will benefit little from tolerance. The balance imposed by the epistatic nature of this relationship is determined by the

cost of fitness to the host. If resistance and tolerance are both costly, the host will produce an animal that favors either tolerance or resistance, but not both. However, if resistance is costly, selection will favor animals with high tolerance and low resistance.

This is the first study that has investigated the heritability of tolerance to Johne's disease. The selection of animals with tolerance to Johne's disease would reduce the severity of the disease in animals that are tolerant, but may result in less selection pressure for resistant animals.

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CHAPTER 3

IDENTIFICATION OF LOCI ASSOCIATED WITH TOLERANCE TO JOHNE'S DISEASE IN HOLSTEIN CATTLE

3.1 ABSTRACT

Johne's disease is a bacterial illness caused by *Mycobacterium avium* subspecies *paratuberculosis* (Map). While the heritability of tolerance has been estimated at 0.09 ± 0.036, the loci associated with tolerance have not yet been investigated. The objective of this study was to identify loci associated with genetic tolerance in cows infected with Map. Feces, ileum, two ileo-cecal lymph nodes, and tissue from the ileo-cecal valve were harvested and cultured for Map from 245 Holstein cows from four dairy herds. Ninety-four cows had a Map colony-forming unit (cfu)/gram(g) of tissue value ≥ 1; 42 of these had fecal cfu/g of feces values ≥ 1, and 8 animals had cfu/gram of feces values that exceeded their tissue cfu/g of tissue. A tolerance index was calculated from average and peak cfus/g of tissue and feces for each animal. The average tolerance index (the average fecal count in colony forming units (cfu_f) of 4 fecal replicates divided by the average tissue colony forming units of 4 tissues, each with 4 replicates (cfu_t)) was calculated for 94 animals whose tissues demonstrated evidence of Map infection. The peak tolerance index was calculated similarly to the average tolerance index using only the highest cfu/g of tissue and the highest cfu/g of feces for each animal. To compute the tolerance index for each animal, for either peak or average tolerance,

the cfu/g of feces + 100 was divided by the cfu/g of tissue + 100. Genotyping of these 94 animals was conducted with the Illumina Bovine SNP50 Bead array. After quality control removal of SNPs, and animal samples, 45,789 SNPs and 90 animals remained. The statistical analysis was conducted using the R statistical environment and PLINK (version 1.04). Tolerance values were treated as a quantitative trait and compared with allele frequencies for each SNP. Strong evidence for association was identified with peak tolerance and a locus on BTA 15 ($p = 1.8\text{x}10^{-7}$, after Bonferroni correction $p = 0.005$), while moderate evidence for association ($p < 3.0\text{x}10^{-5}$) was identified on two adjacent SNPs on BTA 6. The same SNP on BTA 15 showed moderate evidence for association ($p = 2.0\text{x}10^{-6}$) with average tolerance. Two additional SNPs also showed moderate evidence for association on BTA 2 ($p = 3.3\text{x}10^{-5}$), and BTA 1 ($p = 3.3\text{x}10^{-5}$). This is the first study to evaluate and identify genetic tolerance loci with Johne's disease in cattle.

3.2 INTRODUCTION

Bovine paratuberculosis, also known as Johne's disease, is an incurable infectious bacterial disease estimated to be present in 67% of US dairy herds, resulting in annual losses exceeding $200 million US dollars [3,26]. *Mycobacterium avium* subspecies *paratuberculosis* (Map) is the microorganism responsible for Johne's disease in cattle [12,25,26,37]. The low sensitivity of the current diagnostic techniques, the long incubation period (4 to 5 years) until the appearance of clinical signs, and the increasing density of animals in production are some of the major road blocks to controlling the disease [8,10].

Tolerance is a defense mechanism that limits the severity of the damage caused by a pathogen once the animal is infected. Tolerance to Map would result in limited bacterial shedding of animals infected with Map. Genetic selection of

animals that are tolerant to Johne's disease may offer a new opportunity to limit disease transmission minimize, animal and economic losses, and reduce risks to human health.

Loci associated with tolerance to Johne's disease have not previously been investigated. The purpose of this study was to identify loci associated with tolerance to Johne's disease using a genome-wide approach. Such identification could be used to develop a marker-assisted selection program to reduce the severity and the losses caused by Johne's disease in cattle.

3.3 MATERIALS and METHODS

3.3.1 Selection of Animals and Data Collection

Fecal samples for two hundred forty-five Holstein cows from four dairy herds located in New York (herd A), Pennsylvania (herd B and herd C), and Vermont (herd D) were cultured every 3 to 6 months between January 1999 and November 2007 or until the animals were culled for any reason. The disease status of the animals was determined by the number of Map colony forming units (cfus)/(g) of tissue from samples of the ileum, ileo-cecal valve and two adjacent ileo-cecal lymph nodes harvested at slaughter [41, 42].

Tolerance was estimated only in animals where Map cfu/g of tissue was ≥ 1.0 in the ileum, ileocecal valve, or two ileocecal lymph nodes. The disease status of the animals was determined by the tissue results, because of the higher sensitivity of tissue diagnostic testing compared to fecal culture or ELISA diagnostic testing. Animals with tolerance index values lower than one were considered tolerant. Ninety-four (38%) of the 245 animals tested had tissues infected with Map

Table 3.1: Number of animals tested and number of animals infected by herd.

Herd	Number infected	Number tested
Herd A	30	75
Herd B	9	49
Herd C	16	31
Herd D	39	90
Total	94	245

(**Table 3.1**). The mean age of animals was 59 months, with a range of 22.87 to 135 months.

The tolerance index was calculated using either the peak tolerance or average tolerance from the fecal and tissue samples taken at slaughter. The tolerance index was defined as the cfus/g of feces (cfu_f) + 100 divided by the cfus/g of tissue ($cfus_t$) + 100.

Both peak tolerance and average tolerance indexes were used as phenotypic measures for tolerance to Johne's disease and were used to test for an association with the SNPs on the Bovine SNP50 Beadchip.

3.3.2 DNA Preparation and Genotyping

DNA was extracted from 15-40 mg of tissue from each animal using the Puregene DNA extraction kit per manufacturer's instructions (Gentra, Minneapolis, MN). DNA samples were quantified using NanoDrop spectrophotometry, and DNA purity was estimated using the 260/280 ratio. Samples with 260/280 ratios below 1.8 or higher than 2.0 were excluded because of possible protein and or RNA contamination that could compromise the quality of the genotype. Five μg of DNA were diluted to a final concentration of 50 ng/μl. Two hundred fifty ng of

each animal's DNA was genotyped with the Illumina BovineSNP50 Beadchip as described [21]. The Illumina BovineSNP50 Beadchip array contained 55,074 SNPs with a median spacing of one SNP every 35 kb across the bovine genome.

3.3.3 Quality Assurance

Genotypes were derived from data from the Bovine SNP50 Beadchip that was were brought into a single BeadStudio file. Genotypes were identified using a custom genotype SNP cluster file that was based on $\geq$ 2,000 samples from multiple cattle breeds. Population-based association studies are often compromised by false or nonreplicable findings which are partially due to population stratification. To detect and adjust for this possible confounding effect, three methods were used: the multidimensional scaling plot (MDS plot), the quantile-quantile plot (Q-Q plot), and the genomic inflation factor. The MDS plot is a spatial representation of the relationships of the genotypes of the animals in the study and is used to assess and detect the fit of the genotypes of all the animals as one population. Multidimensional scaling analysis (MDS) plotted the genome-wide identity-by-state (IBS) of the SNPs to identify animals with significant genetic variation. The Q-Q plot is a graphical technique for determining if two data sets come from a common distribution. A Q-Q plot is a plot of the quantiles of the first data set against the quantiles of the second distribution. A quantile is the fraction (or percent) of points below the given quantile. That is, the 0.3 (or 30%) quantile is the point at which 30% percent of the data fall below and 70% fall above that value. A 45 degree reference line is also plotted. If the two sets come from the same distribution, the points should fall along this reference line. The greater the departures from this reference line, the

greater the evidence for the conclusion that the two data sets come from different distributions.

The method employed with the genomic inflation factor is that the inflation factor (λ) measures the median distribution of the chi-square statistic from results of a chi-square test done over a set of independent markers and dividing this median by the median of the corresponding (ideal) chi-square distribution. If the results are less or equal to one, the distribution is considered close enough to ideal. If the results exceed one, then an adjustment must be made.

The MDS plot was constructed with 7,250 SNPs, from over 50,000 SNPs to reduce the level of linkage disequilibrium between SNPs. This reduced the correlation between SNPs to less than $r^2 = 0.2$. For these SNPs, the genome-wide IBS pair-wise identities between each pair of animals was computed using PLINK (Version 1.04) [30]. PLINK was also used to compute the Q-Q plots and genomic inflation factor.

3.3.4 Association Analysis

Tolerance to Johne's disease was treated as a quantitative trait based on the tolerance indexes. The whole genome association analysis was conducted using the R statistical environment and PLINK, (Version 1.04) [30]. The likelihood ratio test and the Wald statistical test were used within PLINK to test for an association between each SNP and the tolerance phenotype. Significance for association tests were based on the recommendation of the Wellcome Trust Case Control Consortium where unadjusted p values less than $5\text{x}10^{-7}$ were considered to provide strong evidence of association, and unadjusted p values between $5\text{x}10^{-5}$ and $5\text{x}10^{-7}$ were considered to provide moderate evidence for association [11]. Associations with strong evidence, as defined by the Wellcome Trust Case Control

Consortium, coincide with the associations identified after Bonferroni correction for multiple testing.

3.4 RESULTS

3.4.1 Quantitative Analysis for Association of Loci With Tolerance to Johne's Disease

Animal Quality Assurance

Quality assurance for animal samples revealed that less than 90% of SNP genotypes for two animals were present. Because of the high rate of missing genotypes in these two samples, they were removed from the analysis. In the remaining animals, 98.9% of the genotypes were identified and used in the analysis.

Two additional animals were removed after evaluation with the MDS plot. The MDS plot (**Figure 3.1**) identified two animals from the same herd whose genetic backgrounds differed from the remaining animals in the four herds. The removal of two animals with low genotyping call rates, and two animals with different genetic backgrounds from the remaining animals, left 90 animals for the association analysis. **Figures 3.2 and 3.3** demonstrate that there was no evidence for population stratification for peak and average tolerance after the removal of the four animals described above. The genomic inflation factor for peak tolerance and average tolerance was $\lambda_{GC} = 1.03$ and $\lambda_{GC} = 1.0$, respectively.

Single Nucleotide Polymorphism (SNP) Quality Assurance

Single nucleotide polymorphism quality was assessed prior to the association analysis. SNPs with minor allele frequencies less than 1% (8,131 SNPs), and 1,349 SNPs where less than 90% of their genotypes were present (10% or greater no-call

rate) were removed from the analysis. Monomorphic or non-informative SNPs were also removed, leaving a total of 45,789 SNPs for the association analysis.

Tolerance Index Values

The average and peak tolerance index distributions did not fit a normal distibution (**Figure 3.4**). A log_{10} and a log_2 transformation were performed on the tolerance index values to attempt to normalize their distribution. These transformations did not normalize the tolerance index distributions. Fortunatly the likelihood ratio test used for the association analysis is a robust statistical test that remains valid with a skewed distribution. Peak tolerance index values ranged from 0.27 to 1.07 and average tolerance value ranged from 0.35 to 1.64. The peak and average cfus/g of tissue were highly correlated with peak and average cfus/g of feces ($r^2 = 0,73$ and $r^2 = 0,83$, respectively).

Whole Genome Association Analysis

A whole genome association study was conducted to detect an association of tolerance to Johne's disease with 45,789 SNPs (**Figures 3.5 and 3.6**). Strong evidence for association for peak tolerance was found with a SNP located on BTA 15 approximately 21,000,000 nucleotides from the telomere (unadjusted $p = 1.1\text{x}10^{-7}; p = 0.005$ after Bonferroni correction). **Figure 3.7** illustrates that all Johne's tolerant animals (animals with low tolerance indexes) have one or more B allele of this SNP. The minor allele frequency (MAF) of this SNP was 0.43. The two nearest flanking SNPs were located 28 kb upstream and 29 kb downstream. Neither of these SNPs were associated with peak tolerance (unadjusted $p > 0.03$).

Two aditional SNPs, located 79 kb apart from each other on BTA 6, were found to have a moderate association with tolerance (unadjusted $p = 3.0\text{x}10^{-5}$). The

MAFs for both SNPs were 0.15. The (B) allele was associated with tolerance for the proximal and the distal SNPs on BTA 6. The flanking SNPs on BTA 6 were not associated with peak tolerance (unadjusted $p > 0.01$).

A moderate association ($p = 2.1\text{x}10^{-6}$, $p = 0.09$ after Bonferroni correction) was found for average tolerance to Johne's disease and a SNP on BTA 15. This is the same SNP on BTA 15 that was strongly associated with peak tolerance. Two additional SNPs with moderate evidence for association of tolerance to Johne's disease were located on BTA 2 (unadjusted $p = 3.0\text{x}10^{-5}$ with a MAF of 0.03), and BTA 1 (unadjusted $p = 3.0\text{x}10^{-5}$ with a MAF of 0.45). The A allele of the SNP on BTA 1 was associated with low tolerance index values, representing a Map tolerant animal (**Figure 3.8**). The (B) allele of SNP on BTA 2 was associated with a tolerant animal.

Comparison of animals with the favorable alleles of SNPs on BTA 15 for peak tolerance identified 31 animals that were homozygous for the favored alleles. The mean peak tolerance index values for these animals was 0.63. For the 41 animals that had just one favored allele (heterozygous) the mean peak tolerance index value was 0.83. There were 18 animals that were homozygous for the unfavored allele on BTA 15 and they had a mean peak tolerance index of 0.94.

Two animals were homozygous for for favorable alleles on BTA 6. Their mean peak tolerance index value was 0.53. Twenty two animals were heterozygous for the favored allele, and 65 were homozygous for the unfavored allele with mean tolerance peak values of 0.64 and 0.84, respectively.

A comparison of favorable alleles of SNPs on BTA 15, BTA 1 and BTA 2 for average tolerance, identified 9 of animals that were homozygous for the favored allele at all three SNPs. The mean average tolerance index values for these animals was 0.74. For the animals that were heterozygous for the favorable allele

on BTA 15, the mean average tolerance index value was 0.91. Eighteen animals were homozygous for the unfavored allele and had a average tolerance index value of 1. Thirty-one animals were homozygous for the favored allele on BTA 15 and their tolerance index mean was 0.78.

Heterozygous animals for the favorable allele on BTA 1 (n=41), had a mean average tolerance index value of 0.89, whereas 20 animals homozygous for the favored allele, had an average tolerance index of 0.76. Twenty-nine animals were homozygous for the unfavored allele with an average tolerance index of 0.97.

There were 7 heterozygous animals with a favorable allele on BTA 2. Their mean average tolerance index value was 0.63. Eighty-three animals were homozygous for the unfavorable allele on BTA 2. Their mean average tolerance index was 0.91.

In all cases, the animals with homozygous favorable alleles were more tolerant than those animals that were heterozygous for the favorable allele or homozygous for the unfavorable allele. Animals with favorable alleles at more than ons SNP were more tolerant in general than animals with only one favorable allele, suggesting a possible synergistic effect across loci.

3.5 DISCUSSION

This is the first study to identify loci associated with tolerance to Johne's disease in cattle. To identify loci associated with tolerance, a definition of tolerance to Johne's disease was needed. The definition of the phenotype is a critical aspect of association studies of complex traits. In this study the phenotype of tolerance to Johne's disease was measured using a index that accounted for severity of the disease in animals infected with the bacterium responsible for the disease.

Tolerant animals were defined as animals with high levels of Map in the tissues and lower levels of Map in the feces.

Because tolerance (severity of disease) can only be measured in infected or diseased animals, all animals in this study had detectable levels of Map in their tissues. All infected animals contacted Map in a natural setting and so time of initial infection was not known. To detect if the the age of infection was confounding the tolerance values, the age of infected and uninfected animals was compared within those animals that were slaughtered. There was no difference in the age of these groups. A correlation was calculated between the age of the animal and the quantity of cfus/g of tissue to determine if high cfus/g of tissue were typically found in older animals, and thus primarily a function of age. A positive correlation would indicate that high cfus/g of tissue were a function of age. The correlation, however, was small and negative ($r^2 = -0.09$), indicating that the tissue Map levels were not dependent on age and thus, tolerance was not confounded by the age of Map exposure. A similar calculation was performed for age and the quantity of cfus/g of feces. Again, the correlation was negative ($r^2 = -0.18$) suggestion that as an animal ages and is tissue infected with Map, the animal typically is more tolerant (has a lower tolerance index) to Johne's disease.

There are no perfect measures for identifying and diagnosing animals with Johne's disease. The use of tissue samples to identify Map infected animals reduces the misclassification of animals in early stages of disease as compared with the diagnoses of animals using fecal culture or ELISA [38, 41, 42]. This is not suggesting that tissue diagnosis of animals is a perfect method. It is possible that some animals may have been misdiagnosed.

Complex traits, including most diseases, are distributed as quantitative traits and usually determined by complex genetic and environmental factors,

gene-gene interactions, and gene-environment interactions [17]. In many cases of complex diseases, the infectious status of the animal is characterized as some threshold value [9]. The use of genome-wide association analysis is more powerful than linkage studies for the identification of loci associated with complex traits. The use of the disease phenotype of tolerance as a quantitative trait provides relevant information of phenotypic variation associated with disease that can be used to identify loci associated with the phenotype.

It is not known if Map is involved in either the etiology or in the exacerbation of Crohn's disease. Map is more prevalent in Crohn's patients than in those without Crohn's disease [1]. Selection of animals that are tolerant to Johne's disease would limit the shedding of Map in cattle feces. This in turn would decrease environmental contamination. The presence of high levels of Map in tissue and meat in tolerant animals may represent a second means of Map transmission to humans. It has been shown that highly infected cows with Johne's disease have Map dissemination throughout their muscles and milk. There have been conflicting studies on the efficacy of pasteurization of milk in destroying the viability of Map [24]. Studies to identify levels of cooking necessary to render Map unviable in meat have not been done. Selection of animals that are tolerant to Johne's disease may require additional studies to determine the transmission potential of Map in infected milk and meat that will later serve as human food.

The pathology of Crohn's disease and Johne's disease share some common features. Because of this it has also been suggested that the genes involved in predisposing humans to Crohn's disease may also predispose cattle to Johne's disease. The genes that have been repeatedly identified as predisposing humans to Crohn's disease include: *NOD2, CARD15, IL23R, STAT6* and *SLC22A5* [15,23,31]. These orthologous genes in cattle were not located near the

SNPs associated with tolerance in this study.

The identification of SNPs associated with tolerance to Johne's disease provided a list of positional candidate genes that might be responsible for the association detected in this study. On BTA 15, the SNP that was strongly associated with peak tolerance resides 522 kb from Interleukin-18, (*IL18*) it is a proinflammatory cytokine considered to be a key factor in inflammatory bowel diseases [4]. The BTA 15 SNP is also located 428 kb from the 6-pyruvoyltetrahydropterin synthase gene (*PTS*).

Mutations in *PTS* result in serotonin and catecholamine deficiencies that affect a range of immune responses involving cytokines, lytic activity and antibody production that could be crucial for tolerance to be established in Johne's disease [2,39].

Additional positional candidate genes located within 1Mb upstream from the BTA 15 SNP are: testis expressed gene 12 *(TEX12)*, beta-carotene dioxygenase 2 (*BCO2), PIH1* domain containing 2 gene (*PIH1D2*), translocase of inner mitochondrial membrane 8 homolog B *(TIMM8B)*, crystallin alpha B (*CRYAB*) and heat shock 27 kDa protein 2 (HSPB2). *CRYAB* is a gene associated with regulation of inflammatory process and is a marker for squamous cell carcinoma. It is considered to be a key factor for tumor angiogenesis [7,27,36]. *HSPB2* is associated with programed cell death (apoptosis) [20]. *TEX12* testis-specific expression, associated with expression in the testis as well as functioning as a key element of the synaptonemal complex in meiosis [40]. PIH1D2, is involved in pre-RNA processing [13]. *TIMM8B* mediates the import and insertion of hydrophobic membrane proteins into the mitochondrial inner membrane [28].

There are two positional candidate genes, neural cell adhesion molecule (*NCAM1*) and dopamine receptor 2 (*DRD2*) that are located one Mb downstream

from the SNP on BTA 15. *NCAM1* is associated with bipolar disorder and apoptosis [6,29,46]. *DRD2* is involved in the regulation of inflammatory bowel disease and was suggested as a new potential target for therapy in refractory Crohn's disease patients [19]. Dopamine has an important role in suppressing immune functions by decreasing the proliferation of B and T lymphocytes and has been shown to effect the severity of experimental autoimmune encephalomyelitis [22,32]. Dopamine has also been implicated to induce apoptosis of lymphocytes [5].

No positional candidate genes have been annotated within 1Mb upstream or dowstream from the two adjacent SNPs on BTA 6 that were moderately associated with peak tolerance.

The SNP associated with average tolerance on BTA 2 is located 45 kb from chemokine (C-X-C motif) receptor 4 (*CXCR4*), and 188 kb from the mini-chromosome maintenance complex component 6 (*MCM6*). The inhibition of the activity of *CXCR4* decreases invasion of human colorectal cancer cells *in vitro* [16]. *MCM6* has been associated with lactose intolerance and also with the recurrence of squamous papillary tumors [14,44].

Other positional candidate genes located near the BTA 2 SNP are aspartyl-tRNA synthetase (*DARS*) and UBX domain protein 4 (*UBXN4*). *UBXN4* encodes a protein with a domain involved in diverse cell processes, including endoplasmic-reticulum-associated protein degradation and Alzheimers disease [18].

The SNP on BTA 1 that is moderately associated with average tolerance index values resides within nine positional candidates genes. These include: *PFN2, RN13, COMMD2, TM4SF4, TM4SF1, TM4SF18, GYG1, CPB1* and *AGTR1*.

Profilin 2 gene *PFN2*, is associated in controling polyglutamine protein

aggregation [34]. *COMMD2*, is a COMM domain containing 2 protein whose function is unknown. The transmembrane 4 L six family member 4, 1 and 18 (*TM4SF4*, *TM4SF1* and *TM4SF18*) are a group of transmembrane proteins, that are associated with regulation of cell development, activation, growth and motility [43]. Glycogenin 1 *GYG1*, is over experessed depending the intensity of the exercise and the progress of glycogenolysis [35]. Carboxypeptidase B1 (*CPB1*), is a highly tissue-specific protein and is a useful as a serum marker for acute pancreatitis and dysfunction of pancreatic transplants. It is not elevated in pancreatic carcinoma [45]. Angiotensin II receptor type 1 (*AGTR1*) is associated with thromboembolic disease [33].

3.6 CONCLUSION

The identification of loci associated with tolerance to Johne's disease could be used to select animals to reduce spreading of Map in the environment and reduce the incidence and losses caused by Johne's disease. Future work in the replication of this experiment with a new population and greater number of infected animals will be necessary to confirm these associations and to fine map the loci responsible for them.

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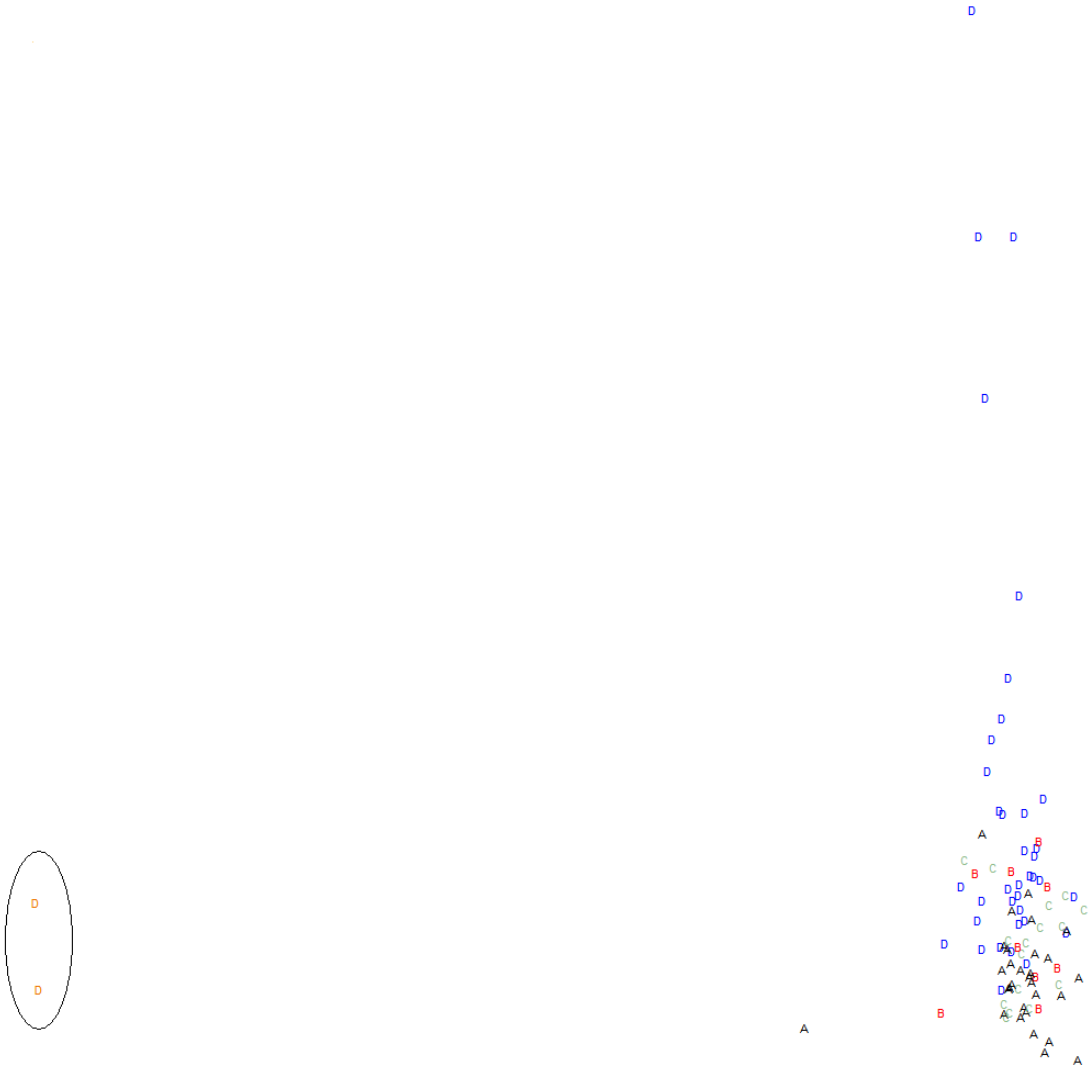

Figure 3.1: Multidimensional scaling (MDS) plot provides a spatial representation of the relationship between animals based on the genotype. Evidence of genetic background differences between 2 animals from herd D (circled), resulted in the removal of these animals from the association analysis. An A denotes for the spatial representation of the genotypes of individual animals from herd A compared with the genotype of the other animals. Animals in all herds are Holstein cattle. Herd A animals from New York, herd B and C from Pensilvania, and herd D from Vermont

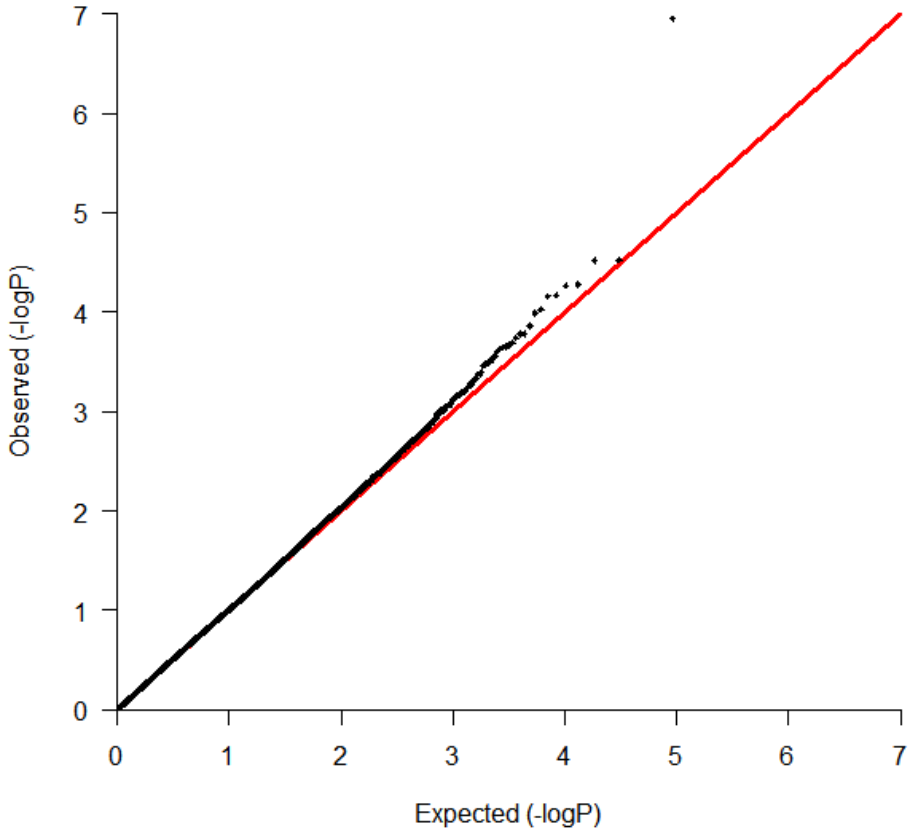

Figure 3.2: Q-Q plot of association analysis with peak tolerance as the phenotype. The observed p values are plotted on the y axis and the expected p values are plotted on the x axix for each SNP. The 45 degree reference line is in red. The black line is composed of black dots that represents the actual plot of significant values for the association analysis of peak tolerance for each SNP in the study. The deviations in this values from the reference line represents the SNPs associated with the peak tolerance phenotype on BTA 15 and BTA 6. The $\lambda_{GC} = 1.03$ for these SNPs and the peak tolerance.

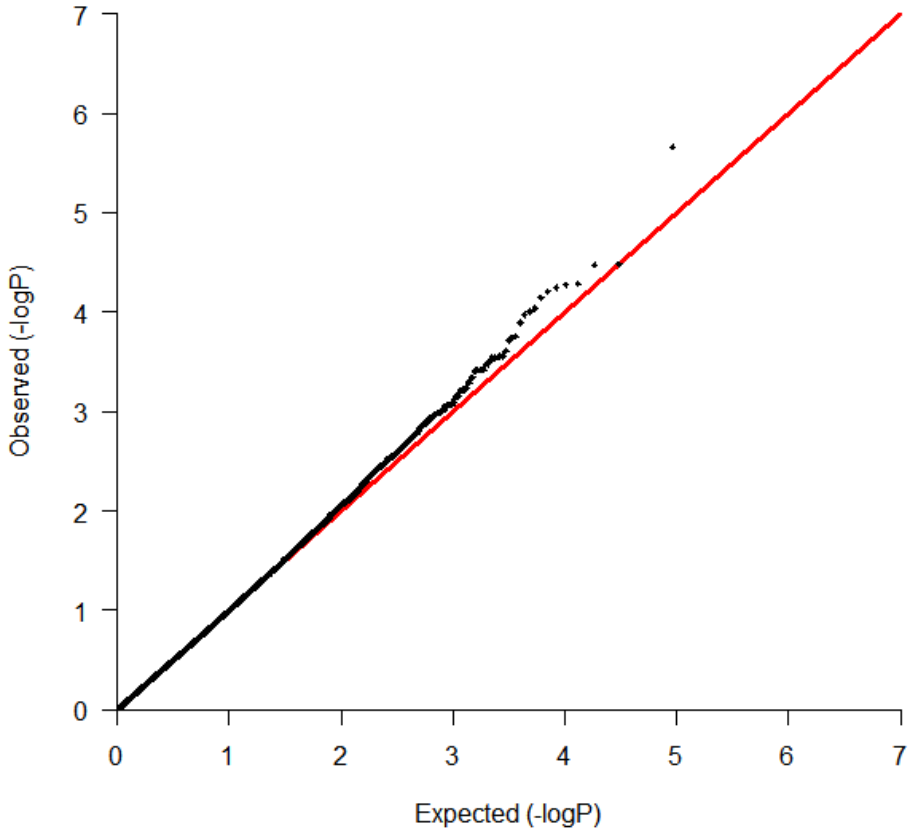

Figure 3.3: Q-Q plot of association analysis with average tolerance as the phenotype. The observed p values are plotted on the y axis and the expected p values are plotted on the x axix for each SNP. The 45 degree reference line is in red. The black line is composed of black dots that represents the actual plot of significant values for the association analysis of average tolerance for each SNP in the study. The deviations in this values from the reference line represents the SNPs associated with the average tolerance phenotype on BTA 15, BTA 2 and BTA 1. The $\lambda_{GC} = 1.00$ for this phenotype of average tolerance with this SNPs is ideal.

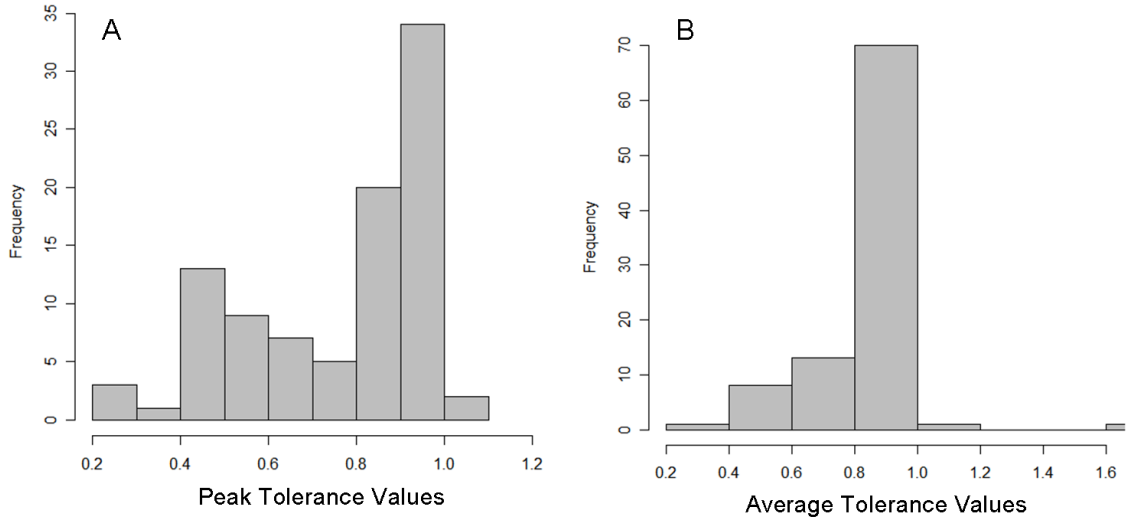

Figure 3.4: Distribution of tolerance values for peak tolerance (A) and average tolerance (B).

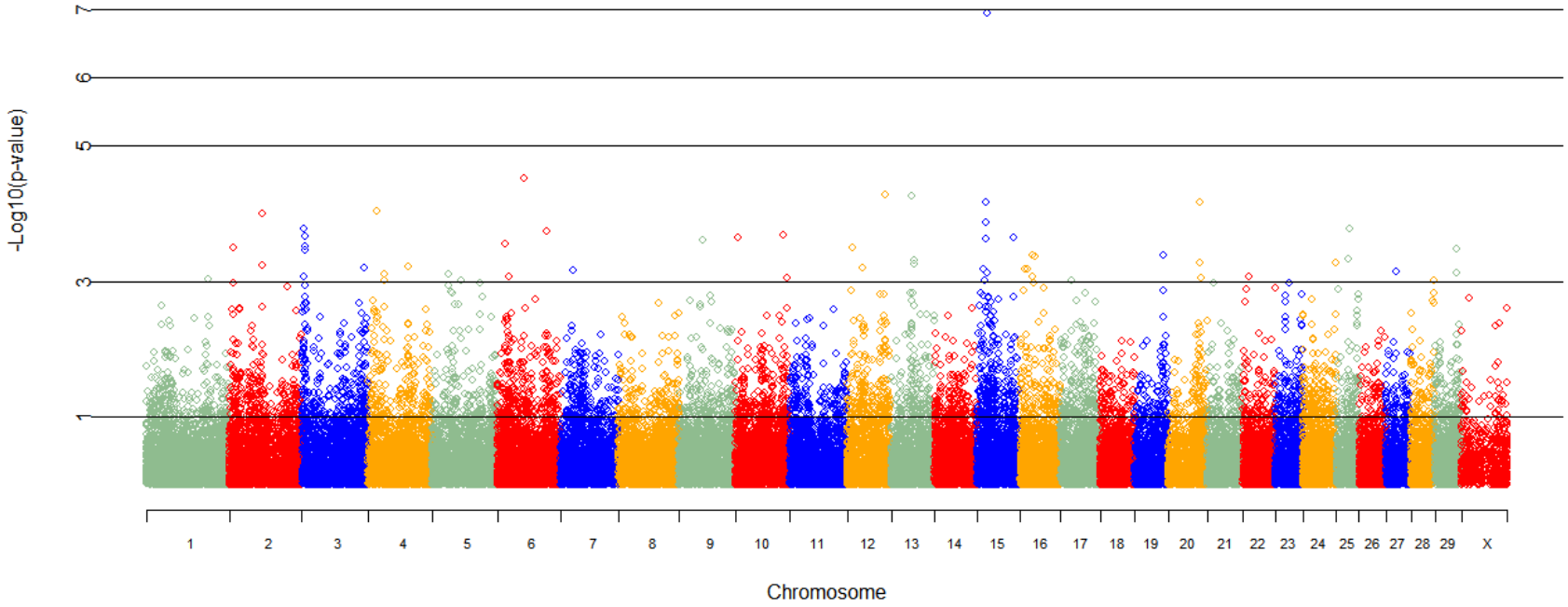

Figure 3.5: Genome-wide association analysis plot of $-log_{10}$ (p-values) for an association of loci with peak tolerance. Chromosomes 1 through 29 and Chromosome X are shown separated by different colors. SNPs located above line 5 were considered significant according with Wellcome Trust Case Control Consortium [11].

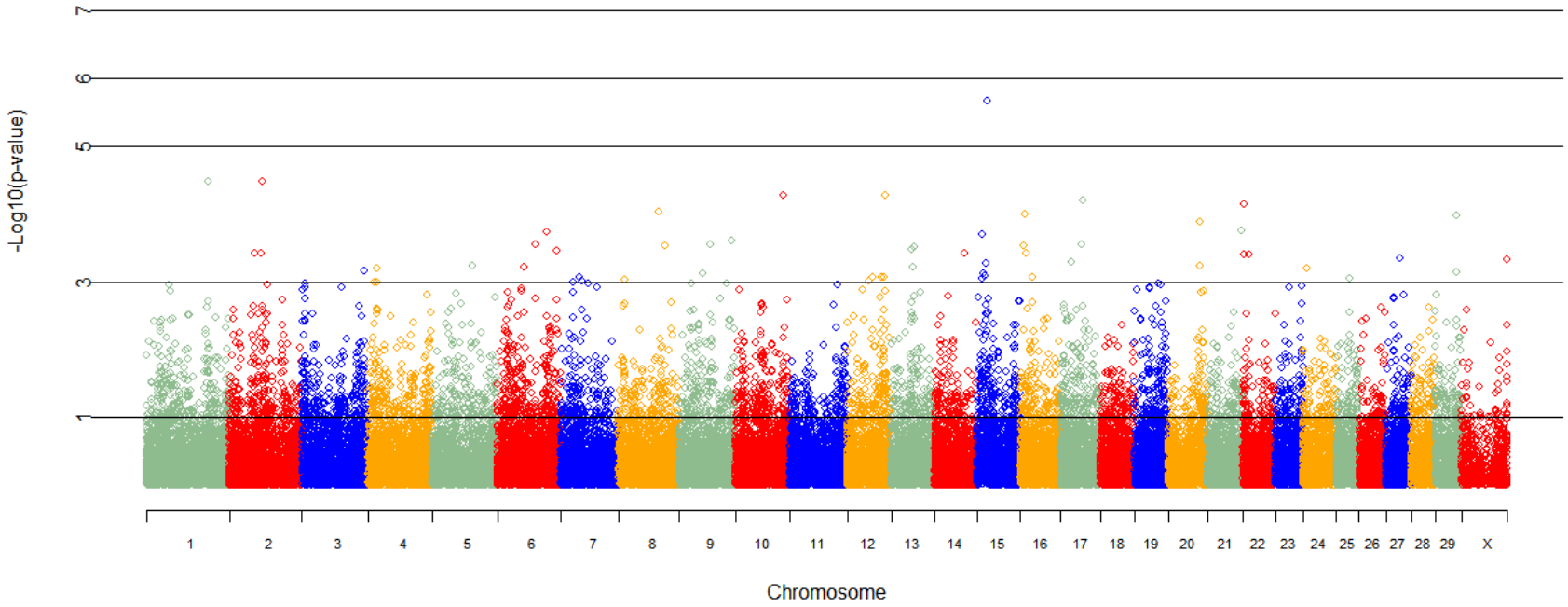

Figure 3.6: Genome-wide association analysis plot of $-log_{10}$ (p-values) for an association of loci with average tolerance. Chromosomes 1 through 29 and Chromosome X are shown separated by different colors. SNPs located above line 5 were considered significant according with Wellcome Trust Case Control Consortium [11].

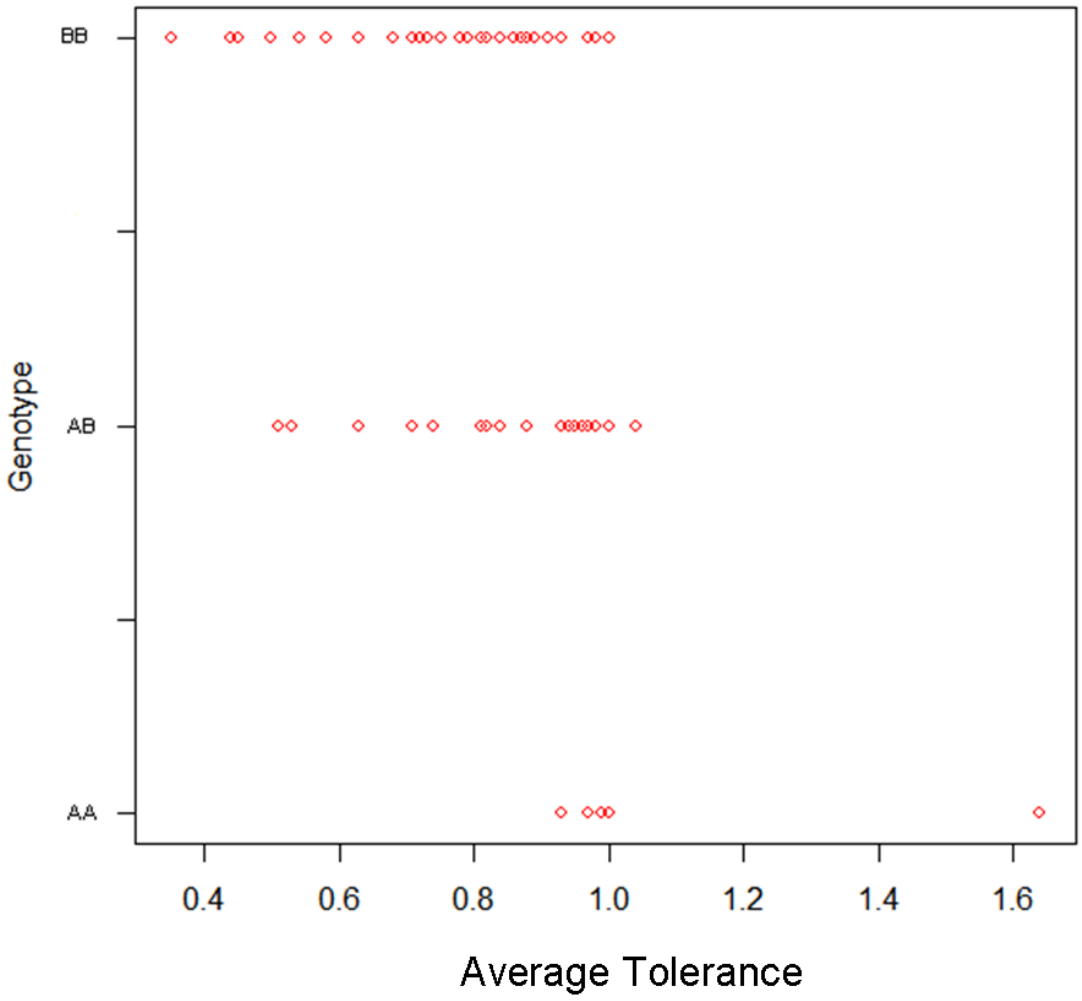

Figure 3.7: Genotype of a SNP on BTA 15 that was moderately associated with average tolerance phenotype ($p = 2.1$ x 10^{-6}). The Y axes represents the genotype AA, AB and BB, and the x axis represents the average tolerance index values for each animal.

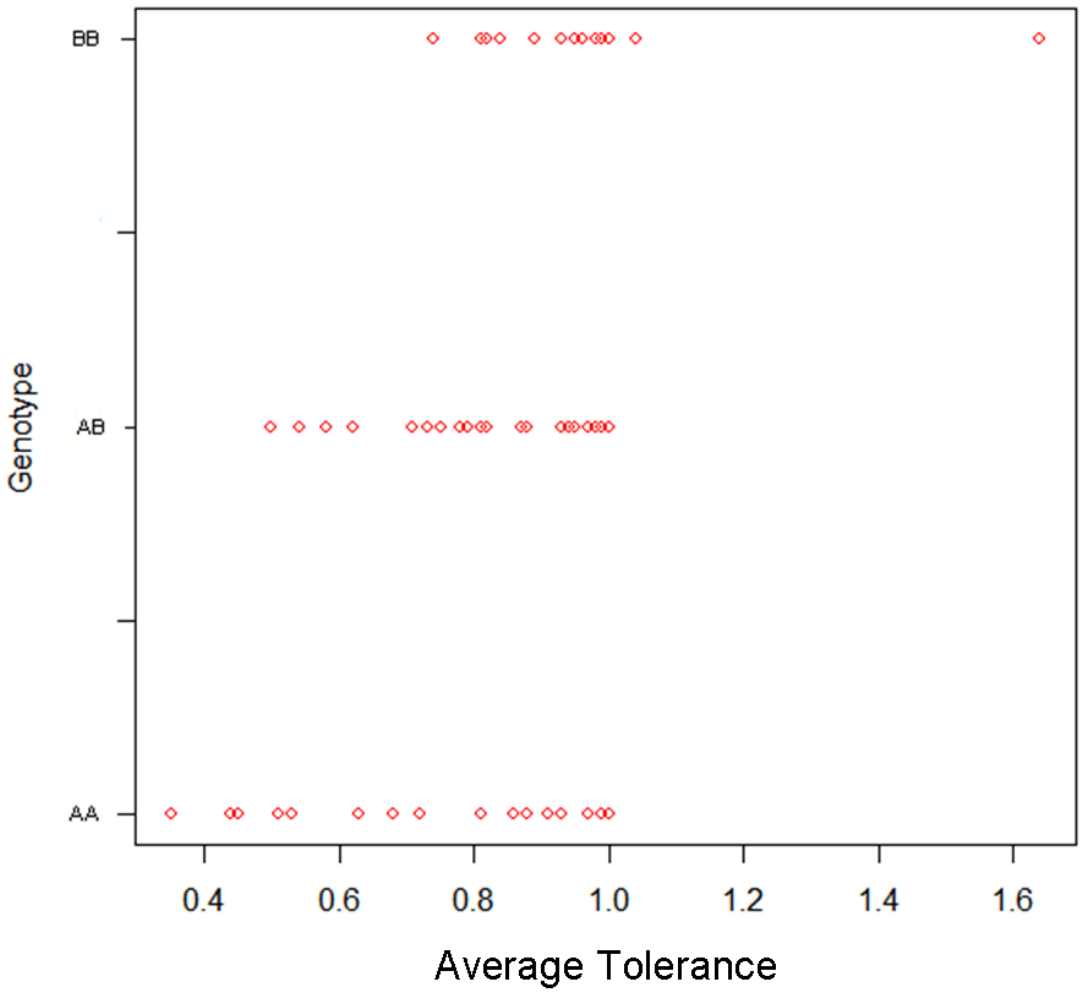

Figure 3.8: Genotype of a SNP on BTA 1 that was moderately associated with average tolerance phenotype ($p = 3.0$ x 10^{-5}) The Y axes represents the genotype AA, AB and BB, and the x axis represents the average tolerance index values for each animal. In this analysis the A allele is associated with tolerance.