Identification of loci associated with tolerance to Johne’s disease in Holstein cattle


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Summary

Johne’s disease, caused by Mycobacterium avium subspecies paratuberculosis (Map), is a fatal disease in cattle. The objective of this study was to identify loci associated with tolerance in cows infected with Map. Tolerance was defined as a cow’s fitness at a given level of Map infection intensity. Fitness was measured by Map faecal cultures, and Map infection intensity was measured by culturing four gut tissues. The quantitative phenotype of tolerance was defined by numerical indexes of cultures of peak (peak tolerance, PT) and average (average tolerance, AT) faecal and tissue Map from 245 Holstein cows. The categorical phenotype was defined as: >100 cfu Map tissue infection, and faecal shedding >75 cfu (intolerant) or <10 cfu (tolerant cows). In 94 cows, Map was identified in >1 tissue, including 44 cows with >100 Map tissue cfu and 36 with >1 faecal cfu. A genome-wide association analysis was performed after filtering, leaving genotypes for 45 789 SNPs in 90 animals for the quantitative phenotype and 16 cases and 25 controls for the categorical analysis of tolerance. rs41748405:A>C (BTA15) was associated with PT (P = 1.12 · 10^-7) and AT (P = 2.17 · 10^-6). Associations were identified with PT and adjacent SNPs ss61512613:A>G and ss61530518:A>G (BTA6) (P < 3.0 · 10^-5), and with AT for ss61469568:A>G (BTA 2) (P = 3.3 · 10^-5) and ss86284768:A>G (BTA1) (P = 3.31 · 10^-5). For the categorical phenotype, an association was found with ss8632653:A>G (BTA6) (P < 5.0 · 10^-5). This is the first study to identify loci associated with tolerance to Johne’s disease.

Keywords Johne’s disease, Mycobacterium avium paratuberculosis, tolerance, whole genome association.

Introduction

Tolerance (t) has been defined by Stowe et al. (2000) as \[ F = a + tl, \] where \( F \) indicates the fitness of the hosts at a given level of tissue infection, \( I \). The fitness of the host when uninfected is the \( y \)-intercept \( a \), and tolerance is the slope of the relationship between fitness (\( F \)) and \( I \) (infection intensity) (Stowe et al. 2000). Infection intensity is impacted by pathogen exposure, host defences and the interaction of the host defences and pathogen virulence. Population differences in tolerance are detected when fitness differs relative to infection intensity. The identification of loci associated with these differences in tolerance is the focus of this study.

Tolerance to Johne’s disease is composed of the infection intensity of the responsible bacterium, Mycobacterium avium subspecies paratuberculosis (Map), and a measure of the change in fitness of the bacterium infected animal (Smyth & Christie 1950). For the purposes of this study, infection intensity was determined by measuring the Map levels in four tissues (ileum, ileo-cecal valve and two ileo-cecal lymph nodes) of each animal. Animals with Map present in one of the four tissues were subsequently evaluated for their level of fitness. The fitness of the Map-infected animals was determined by the amounts of viable Map that were shed.

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into their faeces. Map faecal shedding was chosen as a measure of fitness, as it is associated with increased Johne’s disease severity, decreased milk production, decreased fat and protein content of milk, lower productive life and an increased number of days between pregnancies (Benedictus et al. 1987; McNab et al. 1991; Wice & Gordon 1995; Nordlund et al. 1996; Johnson-Ileaurulundu et al. 2000; Gonda et al. 2007a). Although faecal shedding is also an important measure of the potential transmitting ability of Map to others in the herd, for the determination of tolerance, the amount of Map in the faeces was used in this study as a proxy for fitness. Variation in tolerance was detected when animals differed in the rate of decline of fitness with increasing pathogen levels.

Map faecal shedding was used as a fitness measure to define tolerance in this study, but it is also relevant to the transmission of the disease by environmental contamination to others in the herd. Animals with high levels of Map faecal shedding at the same level of tissue infection are undesirable, because they are intolerant and also because of the higher probability that they will spread disease throughout the herd. Selection of animals that are tolerant indirectly represents selection for animals that pose a lower risk of transmitting Map. This selection strategy may result in animals that are less severely affected after Map infection and may cause a reduction in the prevalence of Johne’s disease due to the decline in Map faecal shedding.

The identification of loci associated with resistance and susceptibility to disease is a concept that has been extensively studied by plant and animal scientists. Tolerance is a concept broadly studied in plants; however, tolerance has yet to be fully examined in animals. Tolerance is a defence mechanism that limits the harm caused by infection, whereas resistance limits pathogen infection (Simms & Triplett 1994; Koskela et al. 2002). The existence of a genetic basis for tolerance has been established by comparing the performance of related plants that are healthy or that have been damaged by pathogens (Simms & Triplett 1994). The application of information about tolerance has been very effective in limiting the negative impact of disease in plants, but has only just begun to be studied in animals (Simms & Triplett 1994; Koskela et al. 2002; Råberg et al. 2007).

The differences in host resistance and tolerance to a pathogen burden have consequences for the evolution of both host and microorganism (Fineblum & Rausher 1995; Roy & Kirchner 2000; Rausher 2001; Miller et al. 2006). Resistance protects the host at the expense of the pathogen, which may result in selection pressure for increased virulence to overcome the host’s defences (Kniskern & Rausher 2001; Woolhouse et al. 2002). In contrast, the evolution of tolerance protects the host from harm while not presenting any direct selective effect to the pathogen. As the pathogen is not selected to increase its ability to infect the host, tolerance does not lead to the antagonistic co-evolution between host and pathogen that occurs with resistance (Rausher 2001; Best et al. 2008).

Tolerance is defined differently in other fields of study. In immunology, tolerance often refers to animals that are immunologically unresponsive. In the study of parasitic infections, tolerance to trypansomes in cattle describes the overall effect of infection on disease severity and host fitness, but does not account for the trypansomone burden (Naessens 2006). Although the definitions of immune- and parasitic-tolerance have concepts in common with our definition of genetic tolerance, the key difference is that tolerance is measured relative to a specific pathogen burden in our definition.

Many factors affect the outcome of a pathogen challenge. Hosts that minimize pathogen infection, due to their resistance to pathogens, may not be as healthy as hosts that have high pathogen levels and are tolerant (Aidoo et al. 2002; Best et al. 2008; Råberg et al. 2009). It is not known how much of the variation in fitness after pathogen exposure is due to tolerance or resistance. Understanding the tradeoffs between host defences underlying resistance and tolerance will provide new information for identifying defence loci associated with Johne’s disease that are the most beneficial for the selection of healthy animals.

Bovine paratuberculosis, also known as Johne’s disease, is an incurable infectious bacterial disease estimated to be present in a minimum of 67% of US dairy herds, resulting in annual losses exceeding US $200 million (Ott et al. 1999; APHIS 2008). The low sensitivity of current diagnostic techniques, long periods (2–12 years) from the time of Map infection until the appearance of clinical signs, and high animal densities in intensive production systems have all been major impediments to controlling the spread of Johne’s disease (Chiodini et al. 1984; Collins et al. 2006). In Johne’s disease control demonstration herds, the utilization of best management practices has reduced disease prevalence, but these practices have not yet been widely adopted across the US (Ferrouillet et al. 2008). Vaccination for Johne’s disease remains controversial as a management tool in the US, as it has not been shown to be effective for preventing infection or transmission of the disease to other animals, and it also interferes with bovine tuberculosis skin testing and serological detection of Map-infected animals (Collins 1996; Muskens et al. 2002). This may be due to the use of a strain of Mycobacterium avium subsp. avium rather than Map in the heat killed whole vaccine that is approved for use in the US (Chiodini 1993). However, vaccines have been reported to reduce faecal shedding and delay the onset of clinical disease, and so increase profitability (Larsen et al. 1978; Kormendy 1994; Van Schaik et al. 1996; Fridrikssonottir et al. 2000; Kalis et al. 2001; Groenendaal and Galligan 2003, Uzonna et al. 2003).

This study explores an additional approach to limit the severity of Johne’s disease through the identification of loci associated with animals that are tolerant to Johne’s disease...
using a genome-wide association approach. The identification of loci associated with tolerance to Johne’s disease could be used to select tolerant animals, which would reduce the severity and losses caused by Johne’s disease in cattle. The primary objective in selecting animals that are tolerant to Johne’s disease is to retain animals that limit the harm that Map causes after infection. It is our hypothesis that after infection with Map, tolerant animals will maintain a higher level of fitness than intolerant animals. Selecting for tolerant animals would also have an indirect secondary effect of reducing Map faecal shedding in infected animals, which would decrease the levels of environmental contamination and lower the likelihood of transmitting the disease to other animals within the herd.

Materials and methods

Selection of animals and phenotype definition

Samples used for this study are the same as those described previously (Settles et al. 2009). Briefly, tissue and faecal samples were harvested at slaughter and cultured for Map from 245 Holstein cows from four dairy herds located in New York (herd A), Pennsylvania (herds B and C) and Vermont (herd D). All of the animals were infected with Map in a natural setting, and the timing of the initial infection was unknown. The infection status of the animals was determined by the presence of Map colony forming units (cfu) as determined by culturing samples taken from the ileum, ileo-cecal valve and two adjacent ileo-cecal lymph nodes (Whitlock et al. 1996, 2000; Pradhan et al. 2008). Culture results from the tissue samples were used to determine infection status because of the enhanced diagnostic sensitivity of this method compared with faecal culture and ELISA diagnostic tests (Collins et al. 2006). Histopathology is also used to detect Map in tissues, but was not used in this study. Gonzalez et al. (2005) reported that histopathology was superior to faecal culture and the ELISA diagnostic tests for detecting Map in tissues, although a more recent study has found that only 6% of the tissues identified to be positive by culture were found to be positive by histopathology (Martinson et al. 2008).

The ages of the Map tissue infected and Map tissue negative animals were evaluated with a Student’s t-test to determine whether there was a difference in age between the animal groups. Tolerance can only be measured in tissue infected or diseased animals, so the analysis of tolerance was limited to animals with a Map cfu of ≥1.0 in at least one of the cultured tissues. Animals that were not Map tissue infected were not included in this study.

Tolerance was defined as the inverse relationship between fitness (measured by Map faecal shedding) and infection intensity (measured by the level of Map tissue infection). Tolerance was defined as both a quantitative trait and a qualitative trait. For the quantitative measurement of tolerance, indexes for peak tolerance (PT) and average tolerance (AT) were calculated for each animal from the peak and average tissue (cfu) and faecal (cfu) Map concentrations obtained from samples taken at slaughter (Equation 1). Four replicates were grown for each faecal sample as well as for each tissue sample. For peak faecal values, the greatest value of the four replicate faecal samples was used for each animal. The peak tissue value was represented by the greatest cfu value for the four tissues evaluated. Average tolerance values were obtained by averaging the four replicate faecal values and averaging the four tissue values (with four replicates for each tissue). To stabilize the variance of the index across the range of values, a constant of 100 was added to both faecal and tissue values. By adding 100 to both the numerator and denominator, the ratio of differences is maintained without inflating the variance due to small differences in tissue cfus. Correlations were estimated to identify the strength of the relationships between average and PT, and between the age of the animal and the quantity of Map in the tissue.

\[
\text{Tolerance} = \frac{\text{faecal}_{\text{cfu}} + 100}{\text{tissue}_{\text{cfu}} + 100}
\]

For the qualitative (case–control) analysis, controls (tolerant animals) were defined as animals that had low peak faecal levels (cfu <10) or no faecal shedding. Cases (intolerant animals) had high peak levels of Map faecal shedding (cfu ≥75). These faecal values were chosen due to the natural clustering of faecal data in the tissue infected animals; one group of animals had high peak faecal shedding (cfu ≥75), the second group had low peak faecal shedding (cfu < 10) and there were no animals exhibiting intermediate values. To avoid potential inconsistencies between levels of tissue infection and levels of Map faecal shedding, only animals that had Map tissue levels ≥100 cfu were used in the case–control analysis. A Student’s t-test was used to identify whether age influenced the categorization of an animal as a case or a control.

DNA preparation and genotyping

DNA extraction and genotyping were as previously described (Settles et al. 2009). DNA was extracted from 15 to 40 mg of tissue from each animal using the Puregene DNA extraction kit as per manufacturer’s instructions (Gentra). DNA samples were quantified using NanoDrop spectrophotometry. Five micrograms of DNA were diluted to a final concentration of 50 ng/μl. Three hundred nanograms of each animal’s DNA was genotyped with the Illumina BovineSNP50 BeadChip as previously described (Matukumalli et al. 2009). The Illumina BovineSNP50 Beadchip assay contained 55 074 SNPs with a median spacing of one SNP every 35 kb across the bovine genome. Genotypes were identified using a custom genotype SNP cluster file developed at the University of
Missouri based on >6000 samples previously genotyped from multiple cattle breeds.

Quality assurance

Population-based association studies are often compromised by false positive or non-replicable findings that are often due to population stratification. To detect and adjust for population stratification, three methods were used: the multidimensional scaling (MDS) plot, the quantile-quantile (Q–Q) plot and the genomic inflation factor ($\lambda_{GC}$). The MDS plot was constructed from 7250 SNPs, condensed from over 50 000 SNPs, with pairwise associations of $r^2 < 0.2$ to reduce the level of linkage disequilibrium between the SNPs. From these SNPs, pairwise identity by state was computed using PLINK (version 1.04) (Purcell et al. 2007) and plotted. PLINK was also used to compute the genomic inflation factor. The Q–Q plot was compiled in the $r$ statistical environment.

Association analysis

A genome-wide analysis was conducted to identify loci associated with tolerance to Johne’s disease as a quantitative trait. Tolerance was also treated as a qualitative trait in a case–control analysis using the R statistical environment and PLINK (version 1.04) (Purcell et al. 2007). The likelihood ratio test and the Wald statistical test were used to test for an association between each SNP and tolerance. The case–control analysis was performed using the standard allelic chi-squared test, and an odds ratio was computed for the association between each SNP and tolerance. The significance of the chi-squared test, and an odds ratio was computed for the association between each SNP and tolerance. The significance values between 5·10$^{-5}$ and 5·10$^{-7}$ were considered to provide strong evidence of association, and unadjusted significance values between 5·10$^{-5}$ and 5·10$^{-7}$ were considered to provide moderate evidence for association (The Wellcome Trust Case Control Consortium 2007). Physical positions at each SNP were expressed relative to the forward strand of the reference genome (BTAU_4.0, http://www.ncbi.nlm.nih.gov/genome/guide/cow/; The Bovine Genome Sequencing and Analysis Consortium et al. 2009).

Results

Map infection status

Ninety-four (38.4%) of the 245 animals tested had at least one tissue infected with Map (cfu ≥1) (Table 1). Of these 94 animals, 41 had Map tissue levels ≥100 cfu, and 35 were Map faecal positive (cfu ≥1). There was no difference in the average age of animals that were Map tissue infected (60 months) and those that were not infected (58 months) ($P = 0.38$). There was a difference ($P = 0.003$) between the average age (51 months) of cases and of control animals (67 months) with an age range of 27–98 months for all tissue infected animals. We did not expect to find that the cases, animals with high faecal shedding, would be younger than the controls, which exhibited low faecal shedding. This difference in age may be due to the small number of cases and controls. Alternatively, this difference may reflect that animals with high levels of faecal shedding may have been more likely to have been infected at a young age, were more vulnerable to infection, or experienced a different course of disease. In the quantitative analysis, a correlation was estimated to identify whether animals that were Map tissue infected tended to have increasingly higher Map tissue levels as they aged. A small positive correlation ($r = 0.02$) with Map tissue levels indicated that age was not a factor in the levels of Map found in the tissues. Index values for PT ranged from 0.27 to 1.07, and AT values ranged from 0.35 to 1.64. The correlation between PT and AT indexes was $r = 0.84$.

Animal and single nucleotide polymorphism (SNP) quality assurance

Quality assurance was conducted to determine whether animals should be removed prior to the genome-wide association analysis due to missing genotypes or population stratification. Two animals had more than 10% of their genotypes missing and were removed from the analysis. In the remaining animals, 98.9% of the genotypes were identified and were left in the analysis. Two additional animals from the same herd were removed after MDS plot evaluation showed that their genetic background differed from the remaining animals in the four herds’ genotypes (see supplemental information). After the removal of the four previously described animals, there was no evidence for population stratification for PT and AT in the remaining animals (see Fig. S1). The genomic inflation factor was $\lambda_{GC} = 1.04$ for PT, $\lambda_{GC} = 1.01$ for AT and $\lambda_{GC} = 1.07$ for the case–control analysis. These low levels of inflation do not require adjustment to the observed test statistic in the association analysis.
A moderate association (flanking SNPs ss86324333 (http://www.ncbi.nlm.nih.gov/snp_retrieve.cgi?subsnip_id=ss86324333) and ss86338219 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_retrieve.cgi?subsnip_id=ss86338219), located 28 kb upstream and 29 kb downstream of rs41748405:A>C, were not associated with PT ($P = 0.82$ and $P = 0.03$ respectively) or AT ($P = 0.92$ and $P = 0.09$ respectively).

On BTA6, two SNPs (ss61512613:A>G and ss61530518:A>G), located within 79 kb of each other and in complete linkage disequilibrium ($r^2 = 1$), were moderately associated with PT (MAF = 0.15, $P = 3.0 \times 10^{-5}$). One SNP, rs43679404:C>T, located between ss61512613:A>G and ss61530518:A>G, was not associated with PT ($P = 0.02$); nor was ss86326791:A>G [sequence NW001495170.2 (http://www.ncbi.nlm.nih.gov/nuccore/194667786)], flanking ss61530518:A>G ($P = 0.187$), or rs43678158:A>G, flanking ss61512613:A>G ($P = 0.01$).

Moderate evidence for an association of AT to Johne’s disease and ss68284768:A>G on BTA1 (MAF = 0.45, $P = 3.3 \times 10^{-5}$) was identified. Flanking SNPs ss65176946:C>T ($P = 0.014$) and ss68339305:A>G [sequence NW001493843.2 (http://www.ncbi.nlm.nih.gov/nuccore/194663906)], $P = 0.57$] were not associated with AT. Moderate evidence was also demonstrated for ss61469568:A>G on BTA2 and an association of AT to Johne’s disease (MAF = 0.03, $P = 3.3 \times 10^{-5}$). The flanking SNPs ss68340919:T>C [sequence NW001494606.2 (http://www.ncbi.nlm.nih.gov/nuccore/194664642)], $P = 0.02$] and ss86295467:T>C [sequence NW001494626.2 (http://www.ncbi.nlm.nih.gov/nuccore/194664713)], $P = 0.002$] were not associated with AT.

The presence of the favourable alleles for all three SNPs associated with AT on BTA1, BTA2 and BTA15 was evaluated to determine how tolerance was affected (Fig. 2). Thirteen different genotype combinations were observed at the three loci. The two groups of animals with the most favourable combination of alleles for the three loci had a mean AT index of 0.53 [homozygous favourable alleles at ss68284768:A (BTA1), and heterozygous for the favourable allele at ss66495698:A (BTA2) and rs41748405:C (BTA15)] and 0.54 (homozygous favourable alleles at ss68284768:A and rs41748405:C and heterozygous for the favourable alleles at ss66495698:G). The group of eight animals with the worst combination of alleles for the three loci (GG-AA-AA for ss68284768, ss66495698 and rs41748405 respectively) had a mean AT index of 1.07. For PT, nine different genotype combinations were observed for ss65152163:A>G (BTA 6), ss61530518:A>G (BTA 6) and rs41748405:A>C (BTA 15). When all three loci associated with PT on BTA6 and BTA15 were considered, one animal was homozygous for the favourable allele at all three loci with a tolerance index of 0.56, and 18 animals were homozygous for the unfavourable alleles at all three loci with a mean PT of 0.94 (Fig. 2). Consistently, animals with favourable alleles at more than one SNP were more tolerant than animals

<table>
<thead>
<tr>
<th>Peak tissue (cfu) value</th>
<th>Peak faecal (cfu) value</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>&lt;10</td>
<td>1–10</td>
<td>2</td>
</tr>
<tr>
<td>&lt;10</td>
<td>75–149</td>
<td>0</td>
</tr>
<tr>
<td>&lt;10</td>
<td>150–300</td>
<td>0</td>
</tr>
<tr>
<td>10–75</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>10–75</td>
<td>1–10</td>
<td>2</td>
</tr>
<tr>
<td>10–75</td>
<td>75–149</td>
<td>0</td>
</tr>
<tr>
<td>10–75</td>
<td>150–300</td>
<td>0</td>
</tr>
<tr>
<td>76–149</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>76–149</td>
<td>1–10</td>
<td>5</td>
</tr>
<tr>
<td>76–149</td>
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<td>11</td>
</tr>
<tr>
<td>76–149</td>
<td>150–300</td>
<td>0</td>
</tr>
<tr>
<td>150–300</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>150–300</td>
<td>1–10</td>
<td>1</td>
</tr>
<tr>
<td>150–300</td>
<td>75–149</td>
<td>5</td>
</tr>
<tr>
<td>150–300</td>
<td>150–300</td>
<td>9</td>
</tr>
</tbody>
</table>

1One animal in this category had a peak tissue (cfu) value of 78, the remaining seven animals had peak tissue values >100.

2All animals in this category had a peak tissue (cfu) of <10.

SNP quality was also assessed prior to the association analysis to eliminate monomorphic SNPs and SNPs with minor allele frequencies (MAFs) <0.01. Monomorphic SNPs (8131 SNPs) and SNPs with a greater than 10% no call rate (1349) were removed prior to the association analysis of tolerance as a quantitative trait, leaving a total of 45 789 SNPs and 90 animals. For the case–control analysis, 41 of the 90 animals had Map tissue levels ≥100 cfu and were included in the association analysis (Table 2). In the smaller group of animals (n = 41), an additional 265 SNPs were filtered due to MAFs <0.01 and monomorphic SNPs, leaving 45 524 SNPs for analysis.

Genome-wide association analysis for tolerance to Johne’s disease as a quantitative trait

A genome-wide association analysis was performed to detect associations between SNPs and tolerance to Johne’s disease using a quantitative measurement of tolerance (Fig. 1; Table 3). Strong evidence for an association with PT was found with SNP rs41748405:A>C, located at approximately 21 Mb on BTA15 (MAF = 0.43, $P = 1.1 \times 10^{-7}$). A moderate association ($P = 2.1 \times 10^{-7}$) with AT was also found for Johne’s disease and rs41748405:A>C. The flanking SNPs ss86324333 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_retrieve.cgi?subsnip_id=ss86324333) and ss86338219 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_retrieve.cgi?subsnip_id=ss86338219), located 28 kb upstream and 29 kb downstream of rs41748405:A>C, were not associated with PT ($P = 0.82$ and $P = 0.03$ respectively) or AT ($P = 0.92$ and $P = 0.09$ respectively).

Distribution of the number of animals that represent each category is given under number (cfu) faecal value is given under the peak faecal (cfu) value, the highest colony forming unit (cfu) value of the tissue levels

<table>
<thead>
<tr>
<th>Peak tissue (cfu) value</th>
<th>Peak faecal (cfu) value</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>150–300</td>
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<td>3</td>
</tr>
<tr>
<td>150–300</td>
<td>1–10</td>
<td>4</td>
</tr>
<tr>
<td>150–300</td>
<td>75–149</td>
<td>1</td>
</tr>
<tr>
<td>150–300</td>
<td>150–300</td>
<td>9</td>
</tr>
</tbody>
</table>
with only one favourable allele, suggesting an additive effect across loci.

**Genome-wide case–control association analysis for tolerance to Johne’s disease**

Forty-one cows with high levels of Map tissue infection (≥100 cfu) were analysed for the case–control study. Of these animals, 16 had low (<10 cfu) Map faecal levels and were classified as tolerant controls, and the remaining 25 animals with ≥100 cfu of Map tissue infection had Map faecal levels between 10 and 75 cfu.

The case–control genome-wide association analysis identified a moderate association with SNP ss86326531: A>G located at 122 Mb on BTA6 using the allelic model (unadjusted \( P = 4.6 \times 10^{-5} \)) (Fig. 1). The MAF in cases was

**Figure 1** Genome-wide association plot of significance values for peak tolerance (PT) (a), average tolerance (AT) (b) and case–control (c) analysis. The results of the genome-wide association analysis for PT in (a), AT in (b) and case–control (c) are shown for chromosomes 1 through 29 and the X chromosome. The results are plotted by the –\( \log_{10} \) significance values on the y axis and the chromosomal location for each SNP tested on the x axis. SNPs located between 4.3 and 6.3 on the y axis provided moderate evidence for association, and SNPs located between 6.3 and 7 on the y axis provided strong evidence for association.

<table>
<thead>
<tr>
<th>SNP</th>
<th>BTA</th>
<th>Position (bp)</th>
<th>Significance (unadjusted)</th>
<th>FA</th>
<th>UA</th>
<th>Homozygous FA</th>
<th>Heterozygous FA</th>
<th>Homozygous UA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ss61512673:A&gt;G</td>
<td>6</td>
<td>51 851 115</td>
<td>2.99 \times 10^{-5}</td>
<td>G</td>
<td>A</td>
<td>PT = 0.53 (2)</td>
<td>PT = 0.64 (23)</td>
<td>PT = 0.84 (65)</td>
</tr>
<tr>
<td>ss61530518:A&gt;G</td>
<td>6</td>
<td>51 772 056</td>
<td>3.00 \times 10^{-5}</td>
<td>G</td>
<td>A</td>
<td>PT = 0.53 (2)</td>
<td>PT = 0.64 (23)</td>
<td>PT = 0.84 (65)</td>
</tr>
<tr>
<td>rs41748405:A&gt;C</td>
<td>15</td>
<td>21 254 061</td>
<td>1.12 \times 10^{-7}</td>
<td>C</td>
<td>A</td>
<td>PT = 0.77 (30)</td>
<td>PT = 0.91 (42)</td>
<td>PT = 1.00 (18)</td>
</tr>
<tr>
<td>ss86284768:A&gt;G</td>
<td>1</td>
<td>120 575 774</td>
<td>3.31 \times 10^{-5}</td>
<td>A</td>
<td>G</td>
<td>AT = 0.63 (30)</td>
<td>AT = 0.83 (42)</td>
<td>AT = 0.94 (18)</td>
</tr>
<tr>
<td>ss61469568:A&gt;G</td>
<td>2</td>
<td>64 266 247</td>
<td>3.32 \times 10^{-5}</td>
<td>G</td>
<td>A</td>
<td>None observed</td>
<td>AT = 0.62 (7)</td>
<td>AT = 0.90 (83)</td>
</tr>
</tbody>
</table>

Table 3 SNPs associated with quantitative phenotype of peak or average tolerance. SNPs associated with peak tolerance (PT) or average tolerance (AT) to Johne’s disease (unadjusted \( P < 5.0 \times 10^{-5} \)), their chromosomal locations, significance levels, alleles and tolerance index values for each genotype. FA represents the favourable allele and UA represents the unfavourable allele for each SNP. Tolerance indexes for genotypes consisting of the homozygous favourable alleles (FA), heterozygous and homozygous unfavourable alleles (UA) along with the number of animals observed (in parentheses) for each category are listed below.
0.32, but this was the most frequent allele (frequency = 0.78) in controls. The estimated odds ratio for the favourable allele of ss86326531:A>G with tolerance to Johne’s disease was 0.13 (95% CI of 0.04–0.36), demonstrating a strong protective effect for this allele with regard to tolerance to Johne’s disease. The detection of additional associations may have been limited by the small number of animals (n = 41) that had high levels of tissue infection.

**Discussion**

Complex traits, including most diseases, are generally considered to be quantitative or threshold traits and are influenced by complex interactions between genes and between genes and the environment (Li et al. 2006). In many cases, the infection status of an animal is characterized relative to a threshold value that the animals need to exceed to present the disease phenotype (Churchill & Doerge 1994). We analysed tolerance using two different approaches, one that defined tolerance as a quantitative trait (ratio of fitness to infection intensity on an individual basis) and a second categorical definition performed to identify loci contributing to an animal’s progression towards clinical disease. For the quantitative approach, allele substitution effects at each SNP locus were tested to determine if associations existed with the tolerance indexes. In the case–control analysis, tissue infection intensity was ≥100 cfu, and the tolerance differences between cases and controls were primarily due to differences in fitness (Map faecal shedding). The use of quantitative and qualitative phenotypes for tolerance identified different loci as being associated with tolerance to Johne’s disease, highlighting the importance of precise definitions of phenotype.

There are advantages and disadvantages to both definitions of tolerance in identifying loci associated with Johne’s disease. Advantages of the quantitative approach include the sensitivity of the index to early disease, the lack of predetermined thresholds for defining tolerance and an increased likelihood that genes associated with the modest effects may be identified. Disadvantages of the quantitative phenotype include that it is unknown whether animals with low levels of Map tissue infection will progress to clinical disease, or if the underlying genetic mechanisms involved in tolerance change with disease progression. For the case–control definition of tolerance, advantages are that it provides a snapshot of animals at later stages of disease, which are more likely to be clinically relevant, and that levels of infection intensity were fairly uniform, with variation being predominantly in fitness. However, disadvantages of the case–control definition are that it most likely does not represent a complete view of the loci associated with tolerance, particularly in early stages of disease; it requires imposed threshold values for tissue infection and faecal shedding; and is more likely to only detect genes of large effect.

The definitions of phenotypes in Johne’s disease are further complicated by the latency of disease and the insensitivity of the most commonly used diagnostic tests. There is no perfect diagnostic test for identifying animals with Johne’s disease. The use of tissue samples to identify Map-infected animals reduces the misclassification of animals in the early stages of the disease compared with diagnoses that are based upon faecal culture or ELISA (Sockett et al. 1992;...
that several genes are involved in tolerance to Johne’s disease. The case–
control association analysis addresses this concern by evaluating only those animals with high 
tissue Map infection levels.

The quantitative analysis of tolerance identified only rs41748405:A>C on BTA15 to have strong evidence for an association with tolerance, whereas, no locus was found to have a strong association with tolerance in the case/control comparison. The different results found between the analyses of these two definitions of phenotype may represent differences in host response to combat early stage disease, which is more accurately measured by the quantitative approach compared with the late stage disease represented in the case/control analysis. The differences in the identified loci may also have been influenced by the different numbers of animals that were present in the case/control analysis (n = 41) and the quantitative analysis (n = 90). We expect that several genes are involved in tolerance to Johne’s disease and that further studies with greater numbers of observations will be required to identify new loci and confirm the loci detected in this study.

Putative positional candidate genes within 500 kb of the SNPs associated with tolerance to Johne’s disease were searched for in the NCBI and UCSC databases. A bovine orthologue of human gene GNA12 located on HSA7 (nucleotides 2,735,238–2,735,532) is adjacent to rs41748405 :A>C in bovine clone AAFC03017275. Targeted GNA12 deletions in mice resulted in chronic intestinal inflammation, further implicating this gene in inflammatory bowel disease (Ahmad et al. 2001). GNA12 has also been implicated in familial hyperaldosteronism type II, the loss of cadherin function and adult human stature (Meigs et al. 2002; Jeske et al. 2008; Soranzo et al. 2009).

Loci previously identified as being either associated or linked with Map infection as measured by the culture of tissues, ELISA or faecal cultures were not associated with tolerance. This was expected, as the mechanisms underlying tolerance and resistance differ. Two genome-wide studies have identified loci for resistance or susceptibility to Johne’s disease, and the results of both of these studies were different from the results of this study, which investigated associations with tolerance. Settles et al. (2009) identified loci on BTA3 and BTA9 that showed strong evidence (P < 5 × 10^{-7}) of association with Map tissue infection and presence of Map in tissue and faeces respectively, in Holstein cows. Settles et al. (2009) also identified SNPs with moderate evidence for association with Map tissue infection (unadjusted P < 5 × 10^{-5}) in regions of chromosomes 1, 5, 7, 8, 16, 21 and 23. Gonda et al. (2007b) undertook a genome-wide linkage study using ELISA, faecal culture or both to diagnose infected animals. They reported a chromosome-wide (P = 0.0319) linkage of resistance to Map on BTA20 in one of the three studied half-sib families.

Several candidate gene studies have also been undertaken to determine whether loci associated with Crohn’s disease or functional candidates are also associated with Johne’s disease. Pinedo et al. (2009a) identified an association with CARD15/NOD2 and Johne’s disease, in contrast to the findings of Taylor et al. (2006). SLC11A1, IFNG, TLR1, TLR2 and TLR4 were evaluated for an association or linkage with Johne’s disease. Of these loci, TLR4 demonstrated a possible linkage and SLC11A1 showed a tendency towards an association, but neither locus provided strong evidence for an association or linkage with Johne’s disease (Mucha et al. 2009; Pinedo et al. 2009b). Similar to the genome-wide studies, none of the candidate genes evaluated was found to be associated with tolerance.

Tolerance is a host defence mechanism that is distinct from resistance. It is not known whether selection for tolerance to Johne’s disease is a better strategy than selection for resistance, but only by identifying the loci associated with tolerance can we compare the efficacy of both approaches. To determine if resistance or tolerance is a more appropriate or effective strategy for selection, the responsible mutations must be identified. One factor that will affect the efficacy of selection in producing the healthiest and highest performing animals will be the level of genetic variation present in the cattle population for the desirable and undesirable alleles for resistance and tolerance, and the proportion of the variance in disease that is explained by these mutations. A second factor that will affect the appropriateness of the selection strategy is the zoonotic potential for Map in humans.

The Centers for Disease Control considers Map to be a zoonotic organism, but it is not clear what level of exposure or viability of Map is required for human health to be affected. A recent study has found that Map is the major cause of co-morbidity in individuals taking anti-TNF therapy (Winthrop et al. 2009). Others have found that killed as well as viable Map is sufficient to elicit an inflammatory Crohn’s-like disease response in mouse knockout models of Crohn’s disease (Singh et al. 2008). It is unknown whether the presence of killed Map is sufficient to cause or enhance disease in immuno-compromised humans. This determination will need to be made before animals are selected for tolerance or are managed to lower morbidity, instead of lowering prevalence alone, as occurs with current vaccines for Johne’s disease. Once the pathogenic potential of Map is more clearly defined, the management and selection of animals infected with Map can be established.

It is not known whether Map is involved in either the aetiology or the exacerbation of Crohn’s disease in humans. Map is more prevalent in Crohn’s patients than in those without Crohn’s disease, and the pathologies of Crohn’s disease and Johne’s disease share some common features (Abubakar et al. 2008). As a result of this, it has been
suggested that the genes involved in predisposing humans to Crohn’s disease may predispose cattle to Johne’s disease. The genes that have provided strong evidence for their association or linkage with resistance to Crohn’s disease in humans include: ATG16L1, C1orf30, CCR6, CDKAL1, IBD5, ICOSLG, IL12B, IL23R, IRGM, ITLN1, JAK2, LRRK2, MST1, MUC19, NKK2.3, NOD2/CARD15, ORMDL3, PTGER4, PTPN2, PTPN22, SLC22A5, STAT3, TNFSF15 and ZNF365 (Klein et al. 2005; Massey & Parkes 2007; Rioux et al. 2007; Barrett et al. 2008; Nakahara et al. 2008). As it has not been shown that a pathogen is responsible for Crohn’s disease, no loci have been studied for their association with or linkage to tolerance to Crohn’s disease. The cattle orthologues of the genes associated with resistance to Crohn’s disease are not located near any of the SNPs that were found to be associated with tolerance to Johne’s disease in this study.

To our knowledge, this is the first genome-wide association study to identify loci putatively associated with tolerance to an infectious disease in cattle. The identification of loci associated with tolerance to Johne’s disease is the first step toward identifying the genes involved in the host defence of tolerance. Once these genes have been identified, they may be used to select tolerant animals that are less harmed by Map infection, reduce the transmission of Map to other animals in the herd, and improve profitability.

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References


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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 MDS plots of the data.

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