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Wingless-type MMTV integration site family member 2 (*WNT2*) gene is associated with resistance to MAP in faecal culture and antibody response in Holstein cattle

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Abbreviated title: *WNT2* is associated with MAP infection in Holstein cattle

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Summary

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a pathogenic bacterium responsible of the lethal Johne's disease in cattle. So far, several GWA studies have been carried out to identify chromosomal regions highly associated with Johne's disease. The aim of this study was to investigate the genetic variability within a pool of 7 genes (*LAMB1, DLD, WNT2, PRDM1, SOCS5, PTGER4* and *IL10*) indicated by former GWA/RNASeq studies as putatively associated with MAP infections and to achieve a confirmation study of association with paratuberculosis susceptibility in a population of 324 German Holstein cattle (162 cases: MAP-positive and 162 controls MAP-negative) using ELISA and faecal cultural tests. SNP validation and genotyping information are provided, quick methods for allelic discrimination were set up and transcription factor binding analyses were performed. The SNP rs43390642:G>T in the *WNT2* promoter region is associated with paratuberculosis susceptibility (p=0.013), suggesting a protective role of the T allele (p=0.043, OR 0.50 [0.25-0.97]). The linkage disequilibrium with the *DLD* rs134692583:A>T might suggest a combined mechanism of action of these neighbor genes in the resistance to MAP infection, which is also supported by a significant effect showed by the haplotype $DLD^T/WNT2^T$ (p=0.047). *In silico* analysis predicted rs43390642:G>T and rs134692583:A>T as essential parts of binding sites for the transcription factors GR and C/EBPβ, GATA-1 respectively, hence suggesting a potential influence on *WNT2* and *DLD* gene expression. This study confirms the region (UMD 3.1: 50639460-51397892) on BTA 4 as involved in tolerance/resistance to Johne's disesase. In addition, this study clarifies the involvement of the investigated genes in MAP infection and it contributes to the understanding of genetic variability involved in Johne's disease susceptibility.

Key Words: *WNT2* gene, MAP infection, Johne's disease, Friesian breed

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a pathogenic intra-cellular bacteria known to be the causative agent for the Johne's disease (paratuberculosis) in cattle and other ruminants. The disease was described for the first time by Johne & Frottingam (1895) as an atypical case of bovine tuberculosis. Nowadays, the pathogenesis of the disease is not completely clarified, but the chronic inflammation of the intestine with granulomatous lesions of the ileum are considered as the main alterations of the intestinal mucosa caused by MAP. Animals with Johne's disease show progressive loss of weight, chronic diarrhea and reduction of productive performances (for a review Purdie *et al.* 2011).

Many reasons prevent the early diagnosis of the disease: the prolonged incubation time (up to 10 years) of MAP, the difficulties to clearly identify the infection status of the animals, the high incidence of subclinical infections and asymptomatic cases. Furthermore, the lack of quick and cost efficient commercially available diagnostic tests with high sensitivity make the application of prophylaxis programs difficult as well as the eradication of MAP from an infected herd very difficult (Beyerbach *et al.* 2001).

It's known that MAP infections are spread worldwide and this condition has a very negative impact on the economy of the dairy industry. Recently, Küpper *et al.* (2013) estimated a reduction of milk yield per day of life in MAP positive cows, whereas in US, the losses were estimated in approximately 200 million USD per year in terms of reduced milk production, limited reproduction efficiency and increased management costs (Ott *et al.* 1999).

Susceptibility to paratuberculosis has been showed to have a genetic component and the heritability in cattle was estimated to range from 0.041 to 0.228 (Koets *et al.* 2000; Mortensen *et al.* 2004; Gonda *et al.* 2006; Hinger *et al.* 2008; Attalla *et al.* 2010; Küpper *et al.* 2012; van Hulzen *et al.* 2012).

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Despite many efforts done and genetic approaches (including microsatellite genotyping, whole genome scanning for quantitative trait loci, SNP arrays) have been attempted (for a review Purdie *et al.* 2011), the identification of the genetic part contributing to the phenotypic variance of MAP susceptibility is still not clear.

However, independent genome wide association studies (GWAS) accomplished in the last years proved that only a restricted number of chromosomal regions carry strongly significant SNP involved in Johne's disease (table 1). Although potential candidate genes have been indicated in each of these studies, so far no further investigation including characterization of the genes in Holstein cattle has been performed.

The understanding of the loci associated with susceptibility/resistance to disease is fundamental to evaluate them into breeding schemes and to eradicate the disease. Therefore, the aim of this study was to investigate the genetic variability within a pool of genes putatively associated with MAP infection and accomplish a confirmation study of association with paratuberculosis susceptibility in a population of German Holstein cattle classified as MAP-positive and MAP-negative using both ELISA and faecal cultural results.

Material and methods

Sample collection, Nucleic Acid Isolation and Diagnostic Tests

A total of 324 German Holstein cows from 15 different farms located in Thuringia (Germany) were considered in this study. To eliminate potential stratification factors a case-control study was designed. Therefore 162 faecal culture positive (AVID, 2007) animals (age >24 months) were chosen as cases, whereas 162 faecal culture negative animals from the same farm, from the same sire and at the same age were used as controls. Blood samples were collected to isolate the DNA according to the procedure described by Montgomery & Sise (1990). DNA concentration and

OD$_{260/280}$ ratio of the samples were measured with the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Antibody levels were determined by using a commercially available ELISA test (CATTLETYPE® MAP Ab, Labor Diagnostik Leipzig, Germany) according to manufactures information.

SNP selection and PCR conditions

Six genes falling within chromosomal regions indicated as highly associated with MAP resistance/susceptibility were investigated for genetic variability. The NCBI Reference SNP data base (http://www.ncbi.nlm.nih.gov/projects/SNP/) was used as tool for the identification of SNP to be genotyped in the MAP tested German Holstein population.

The complete list of the SNPs, the corresponding genes, the positions and the genotyping information are reported in table 2 taking as reference the UMD 3.1 sequence assembly. RNA-Seq data analysis from a current project indicated interleukin-10 (*IL10*) as significantly upregulated during early MAP infection (data not shown), therefore *IL10* gene was further included in the analysis for SNP discovery and genotyping (table 2). To reach this goal, the five exons and the intronic regions of the *IL10* underwent re-sequencing for test samples.

For genotyping DNA fragments ranging from 103 bp to 402 bp were amplified by PCR and digested by specific restriction endonucleases. A typical PCR reaction mix (25 μl) comprised: 50 ng of genomic DNA, 1X PCR Buffer (Promega, Madison, WI, USA), 2.5 mM MgCl$_2$, 5 pmol of each primer, dNTPs each at 200 μM and 1 U of *Taq* DNA Polymerase (Promega). PCR was performed under the following thermal conditions: 95°C for 4 min, 35 cycles at 95°C for 30 s, 56°C for 30 s (with the exception of the protocol run for the SNP rs136770416 whose annealing was set up at 50°C), 72°C for 30 s, and the final extension at 72°C for 5 min. Product specificity was confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis.

Test samples and genotyping

A confirmation test of genetic variability was preliminarly run on 28 German Holstein (14 MAP+ and 14 MAP-) belonging to 6 different farms to validate the chosen SNP. For the negative SNP, the confirmation test was extended to further 48 German Holstein (24 MAP+ and 24 MAP-).

RFLP protocols were set up for each SNP. Digestion of 17 μl of each PCR amplification was accomplished with 10 U of endonuclease (Thermo Fisher Scientific Inc.) for 16h at 37°C. The digestion products were analysed directly by electrophoresis in 2% agarose gel in 1X TBE buffer and stained with ethidium bromide. The samples with missing genotypes were amplified and digested at least twice before the exclusion to the statistical analysis.

In silico Analysis of Transcription Factors Binding Sites

SNP *DLD* rs134692583:A>T; *WNT2* rs43390642:G>T and *IL10* AC_000173:g.3625A>G were analyzed for potential transcription factor binding sites applying the online tool TFSEARCH which is based on the TRANSFAC database (Heinemeyer *et al.* 1998). Transcription factors with predicted binding scores of ≥75 for each allele were included in the analysis (max. score = 100). For each SNP, major and minor alleles including the flanking 15 nucleotides upstream (5') and downstream (3') were analyzed.

Statistical Analysis

The allele frequency and Hardy-Weinberg equilibrium ($\chi 2$ test) were calculated by means of PopGene software *Ver 1.31* (University of Alberta, Canada). SAS system software (SAS 9.1, Institute Inc., Cary, USA) was used to estimate differences between the allele frequencies of the different polymorphisms in the investigated genes between cases and controls. Analyses were done

by Fisher's exact test using three different phenotypes: (1) faecal positive/negative, (2) ELISA positive/negative, and (3) faecal or ELISA positive/negative. According to the same scheme odds ratios (ORs) were calculated for the minor allele at each SNP by SAS system software considering 95% confidence intervals (CI). A test of H_0 for OR=1 was calculated by Fisher's exact test. All tests were two-tailed considering p-values <0.05 as significant.

The linkage disequilibrium parameters (D' and r^2) and the haplotype frequencies for the SNP located on chromosome 4 (rs211391398:C>T; rs134692583:A>T and rs43390642:G>T) were estimated with Haploview software according to the model proposed by Wang *et al.* (2002).

Results

Genetic variability occurred within a group of six genes (*LAMB1, DLD, WNT2, PRDM1, SOCS5* and *PTGER4*) indicated as putative candidates for the resistence/susceptibility to MAP infection. Eight SNPs falling within these genes were chosen from NCBI Reference SNP data base for the genotyping of infected and un-infected animals. *In silico* analysis indicated that these SNPs were responsible for amino acid changes (table 2) or had a potential effect on the transcriptional regulation (either enhance or repress) because the affected nucleotide changes putative binding sites for gene transcription factors (table 3).

Four out of eight SNPs (*LAMB1* rs43388824:A>G; *PRDM1* rs136770416:A>C; *SOCS5* rs134378401:A>C; *PTGER4* rs41944920:C>G) were monomorphic in our test samples (38 MAP+ and 38 MAP-). Therefore the total population of German Holstein (162 cases and 162 controls) was genotyped for the remaining four (*LAMB1* rs211391398:C>T; *DLD* rs134692583:A>T; *WNT2* rs43390642:G>T; *PRDM1* rs136669229:A>C) polymorphic sites plus an additional SNP found in *IL10.* In fact, the comparison of the *IL10* sequences showed two new SNPs (g.1309C>T at the intron 2 and g.3625A>G at the intron 4; numbering is relative to the EMBL acc. no. AC_000173 used as reference) never reported in NCBI data base. The second polymorphic site is located only

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14 bp upstream of exon 5 and it might potentially affect GATA binding sites, therefore it was chosen for the genotyping of the complete population by the set up of a PCR-RFLP method.

The restriction patterns for each of the digestion protocols are reported in the figure 1. Briefly, the transition rs211391398:C>T in *LAMB1* is restricted in 2 fragments for the TT samples (238 bp and 164 bp), unrestricted (402 bp) for the CC genotype, whereas the heterozygote pattern is: 402bp, 238 bp and 164bp. A similar pattern for the transversion rs134692583:A>T is present for the gene *DLD*. The genotype AA is characterized by 2 fragments of 103 bp and 65 bp, the TT genotype shows an undigested band of 168 bp and the heterozygous sample results in 3 fragments (168 bp, 103 bp and 65 bp). The transversion rs43390642:G>T at the *WNT2 locus* shows the following restriction pattern: TT (167 bp), GT (167 bp, 95 bp and 72 bp), GG (95 bp and 72 bp).

The restriction pattern for the transversion rs136669229:A>C in the *PRDM1* gene is characterized by 2 fragments for the AA genotype (103 bp and 39 bp), 3 fragments (75 bp, 39 bp and 28 bp) for the CC and 4 fragments (103 bp, 75 bp, 39 bp and 28 bp) for the AC genotype.

A similar pattern for the transversion AC_000173:g.3625A>G is detected at *IL10* gene. The genotype AA shows 2 fragments of 282 bp and 71 bp. The band 282 bp long is further digested in 2 fragments of 220 bp and 62 bp for the GG genotype. The heterozygous shows 4 fragments of 282 bp, 220 bp, 71 bp and 62 bp. The last two bands have a difference in size of only 9 bp and they were not discriminated on the gel. Therefore, these bands appear as unique for the genotypes AG and GG (Figure 1, lines 22 and 23).

The genotype distribution and the allelic frequencies are reported in the table 4. Missing genotypes vary between 0.3% (*IL10*) and 7.7% (*DLD*). Chi-square values indicated no evidence of departure from Hardy-Weinberg equilibrium in the total population for all the analyzed SNP. The distribution of genotypes according to the phenotypic test (faecal and ELISA) are provided in table 5. Differences in the frequencies of rs43390642:G>T located in the promoter region of *WNT2* were observed in infected animals compared to healthy controls (table 5). The SNP rs43390642:G>T is

associated with paratuberculosis susceptibility using both faecal (p=0.035) and ELISA (p=0.049) diagnostic tests. Association (p=0.013) was also found when a more restrictive phenotype was used (faecal or ELISA positive/negative). In the MAP positive group, the frequency of the rarer T allele of the rs43390642:G>T polymorphism was 0.04 (faecal/ELISA), whereas in the controls it was 0.08. The OR value (p=0.043, OR 0.50 [0.25-0.97]) suggested a protective effect of the minor allele in MAP infection. In contrast, no associations were observed for the other investigated SNP with the exception of rs134692583:A>T in *DLD* gene, whose odd ratio showed a significant effect (p=0.046, OR 0.58 [0.34-0.99]) for the minor allele (T) when faecal and ELISA tests were matched together.

A D' value of 0.968 and an r^2 value of 0.561 suggested a linkage disequilibrium between the 2 SNP (*DLD* rs134692583:A>T and *WNT2* rs43390642:G>T), which formed one haplotypic block covering a region of about 750 kb. The analysis allowed us to detect three haplotypes: AG, TT and TG whose frequencies were 0.894, 0.057 and 0.047 respectively. The χ^2 test run for each haplotype showed a significant association (p=0.047) of the haplotype TT with the resistance to MAP infection.

Discussion

The most recent approach to understand which chromosomal region is involved in MAP susceptibilty is represented by GWAS. So far, several studies have been carried out (Settles *et al.* 2009; Minozzi *et al.* 2010; Pant *et al.* 2010; Kirkpatrick *et al.* 2011; Zanella *et al.* 2011; Minozzi *et al.* 2012; van Hulzen *et al.* 2012) and a limited number of positional candidate genes have been indicated (table 1). Despite this information, little investigation within the genes more or less biologically involved with MAP infection and falling in such regions has been carried out. Our study focused on the variability of 7 genes (*LAMB1, DLD, WNT2, PRDM1, SOCS5*, *PTGER4* and *IL10*) indicated from independent genome wide and RNAseq studies as potentially associated with MAP infection.

Laminin β-1 (*LAMB1*), dihydrolipoamide dehydrogenase (*DLD*) and wingless-type MMTV integration site family member 2 (*WNT2*) genes are located on chromosome 4 spread over a DNA region of about 2Mbp and in the surrounding (~1 Mbp) of the SNP ss66537488:C>T indicated by van Hulzen *et al.* (2012) as associated with paratuberculosis susceptibility by GWAS.

Laminin is a biologically active protein and it is an important structural component of the basement membrane, mediating the interactions between cells and matrix. It was described as one of the molecular determinants involved in the adherence of MAP to epithelial cells (Pethe *et al.* 2001). In human, 3 independent GWAS evidenced the controversial role of *LAMB1* in relation to Crohn's disease (Barrett *et al.* 2009; McGovern *et al.* 2010; van Sommeren *et al.* 2011). Although van Hulzen *et al.* (2012) indicated this gene as putatively involved in Johne's disease, our association study using faecal, ELISA and the combination of both diagnostic test did not confirm such role (table 5). Moreover, the values of OR (range 0.87-1.33) gave no evidence of protective/susceptible allele effect for *LAMB1*. This result is in line with the findings of van Sommeren et al. (2011). Furthermore, our data showed that although located within a DNA fragment of 2Mbp, *LAMB1* rs211391398:C>T does not belong to the same haplotype block as *DLD* rs134692583:A>T (*LAMB1, DLD* - r^2: 0.002) and *WNT2* rs43390642:G>T (*LAMB1, WNT2* - r^2: 0.009), thus suggesting the probable existence of a recombination hot-spot among the first and the last two genes.

The *DLD* gene codes for an enzyme called dihydrolipoamide dehydrogenase. Several recent GWAS report *DLD* as a putative candidate gene for resistance to both human (Barrett *et al.* 2009; McGovern *et al.* 2010) and bovine (van Hulzen *et al.* 2012) mycobacterial infection. This enzyme is involved in many biological pathways related to the energetic metabolism, including the degradation of essential amino acids. The different aptitude to use these nutrients as a result of genetic differences in *DLD* was proposed as a possible solution for the different development of the clinical stage of Johne's disease among infected animals (van Hulzen *et al.* 2012). Our investigation

was focused on the SNP rs134692583:A>T located only 8 bp upstream of exon 1. The association study with MAP infection data showed controversial results. The Fisher's exact test did not confirm the association with all the available phenotypes (table 5). However, the OR (p=0.046; 0.58 [0.34-0.99]) in restrictive phenotype conditions gave evidence of a protective effect of the T allele. The analysis of the putative transcription factor showed that this SNP is responsible of the alteration of several binding sites (table 3). In particular, C/EBPβ is an important regulator of cytokine expression (Cloutier *et al.* 2009), whereas GATA-1 is a potent suppressor of T_h1 associated genes like interferon-γ and chemokine receptor-3 (Sundrud et al., 2005). Both sites are putatively active only in presence of the T allele with a binding allele score of 76.0 and 75.5% respectively, hence suggesting higher transcriptional activation of the *DLD* gene.

The wingless-type MMTV integration site family member 2 (*WNT2*) gene, shown to be significantly associated with MAP infection (p=0.013-0.049) using all the available phenotypes, belongs to a family of structurally related genes (*WNT*) that encode glycoproteins and extracellular signaling molecules. Abnormal *WNT* signaling is linked to a range of diseases, especially cancer. The best-understood *WNT*-signaling pathway goes through the activation of the nuclear functions of β-catenin, which leads to changes in gene expression which influences cell proliferation and survival (Moon *et al.* 2004). Abnormal proliferation of fibroblasts in animals affected with Johne's disease is a key feature in the granuloma formation, consisting of chronic inflammatory cells which include macrophages, giant cells, lymphocytes, plasma cells and fibroblasts that deposit collagen and extracellular matrix (ECM) proteins to create a dense fibrous region similar to a capsule (Ackermann, 2013). Fibroblast proliferation and collagen synthesis are crucial in the repair of injured tissue associated with inflammatory lesions (for a review Flavell *et al.* 2008). *WNT*/β-catenin signaling is activated in this process (Cheon *et al.* 2004). In fact, the injection of soluble *WNT* inhibitor into adult mice inhibits intestinal cell proliferation and suggests the possibility of using *WNT* activators to regenerate gut epithelium as adjuvant therapy in inflammatory bowel

disease (Kuhnert *et al.* 2004). Therefore, this suggests a possible role of the *WNT2* gene in Johne's disease. Our investigation was focused on the SNP rs43390642:G>T which is located in the promoter region of *WNT2* gene. The significant association was confirmed by Fisher's exact test and further validated through the OR values (table 5) which suggested a protective role of the T allele in MAP infection. The presence of thymine is responsible for the creation of a putative consensus sequence for a glucocorticoid receptor element (GR). Glucocorticoids have a vast array of functions within the body, including the potent suppression of immune response and inflammation. Their beneficial use as drugs in the treatment of human chronic inflammatory bowel diseases (IBD) were first recognized nearly 60 years ago (Truelove & Witts, 1954). The major anti-inflammatory effects of glucocorticoids appear to be due largely to interaction between the activated GR and anti-inflammatory genes such as annexin-A1, interleukin-10, and the inhibitor of NF-kB (Hayashi *et al.* 2004). Such mechanism of interaction was well elucidated by Li *et al.* (2003) using a mouse mammary tumor virus (MMTV) promoter, which also characterizes the *WNT2* gene. Briefly, glucocorticoids inhibit expression of adhesion molecules and trafficking of inflammatory cells to target tissues (Hayashi *et al.* 2004), this might explain the protective role of the T allele for the SNP rs43390642:G>T in *WNT2* promoter.

This SNP resulted in linkage disequilibrium with the polymorphic site found in *DLD* promoter. The haplotype TT was associated (p=0.047) with the resistance to MAP infection, but it was less significant than the *WNT2* alone, hence suggesting that the existence of a cooperative action of these genes mediated by the activation of their specific transcription factors has to be deeply investigated. Functional studies are necessary to clarify the influence of C/EBPβ, GATA-1 and GR transcription factors on *DLD* and *WNT2* gene expression, however the potential positive interaction between neighbor genes might partially explain the "infinitesimal" effect expected from these loci in the control of such complex trait.

PR domain-1 (*PRMD1*) is a zinc finger-containing transcriptional repressor of beta-interferon. Our investigation on two A>C transversions responsible for an amino acid change within the *PRDM1* gene did not confirm any association with MAP susceptibilty. However, recently a rare allele identified in *PRDM1* was associated with Crohn's disesase in humans (Ellinghaus *et al.* 2013). Functional studies reported by the same authors showed that this rare risk allele led to increased peripheral blood lymphocytes (PBL) expression of the adhesion molecule L-selectin, increased $CD4^+$ and $CD8^+$ T-cell proliferation, IFN-γ secretion, and up-regulation of activation markers (Ellinghaus *et al.* 2013). Each of these factors may contribute to the pathogenic role of *PRDM1* in this disease.

Suppressor of cytokine signaling (*SOCS*) family of proteins are functionally closely related to interleukins. Cytokine signaling is in fact negatively regulated by *SOCS* proteins. We genotyped a SNP in *SOCS5* gene which is reported in NCBI dbSNP as responsible of amino acid change rs134378401:A^{tyr}>C^{Asp}, however this polymorphic site was monomorphic in German Holstein population. However, *SOCS 5* remains functionally interesting because it is preferentially expressed in T_h1 cells which promote cell-mediated effector responses to eliminate intracellular pathogens (Seki *et al.* 2002).

Prostaglandin E receptor 4 (*PTGER4*) has been identified in human as a Crohn's disease candidate gene by Libioulle *et al.* (2007) and Barrett *et al.* (2009). The same indication was reported after GWAS in Holstein cattle (Kirkpatrick *et al.* 2011). Our confirmation study was carried out on a SNP located at the exon 3 and reported as responsible for an amino acid change rs41944920: C^{Leu}>G^{Val}. However, this SNP was not polymorphic in the German cattle population. Also the role of *PTGER4* related to MAP infection has to be taken in great consideration. Recently in human, Glas *et al.* (2012) demonstrated the strong Crohn's disease association of 2 SNP (rs4495224:A>C and rs7720838:G>T) as part of binding sites for NF-kB and XBP1, suggesting that these transcription factors may modulate *PTGER4* gene expression. Therefore, future investigations

are strongly required also in bovine to clarify the role of *PTGER4* as candidate gene for paratuberculosis susceptibility.

IL10 is classified as a class-2 cytokine. Recent studies of gene expression indicated a greater up regulation of *IL10* in cow monocytes after 2 hours of infection with MAP (Weiss *et al.* 2005). The intronic SNP AC_000173:g.3625A>G within *IL10* is putatively responsible for the alteration of GATA factors binding sites (table 3). This family of transcription factors plays a key role for cytokine gene expression by T_h2 cells (Zheng & Flavell, 1997). In addition, intron GATA binding sites in the *IL4* gene were proved to be essential for acting both as transcriptional enhancer and demethylation factor (Hural *et al.* 2000). Despite these biological functions, the result of the association of the SNP g.3625A>G with both faecal and ELISA tests did not confirm a role of *IL10* in the susceptibility to MAP infection (table 4).

Although the frequencies of minor alleles for all the investigated SNP are low and genotyping larger sample sizes might better elucidate their role in the susceptibility to paratuberculosis, many studies report that low-frequency and rare variants are involved in the etiology of complex traits (Bodmer & Bonilla, 2008; Gibson, 2011). Furthermore, even for diseases with a strong genetic component, the identified common variants usually only explain a small portion of the total genetic heritability. For instance, in a study of Crohn disease, >30 loci were identified, but they explain <10% of the overall heritability (Barret et al. 2008).

In conclusion, this study confirms the region (UMD 3.1: 50639460-51397892) on chromosome 4 as a susceptibility *locus* in Johne's disesase. *WNT2* is significantly associated with MAP infection on the basis of both diagnostic systems faecal culture and ELISA tests. The T allele at the rs43390642:G>T locus showed a potential protective role against paratuberculosis as part of binding sites for a glucocorticoid receptor element, suggesting that this transcription factor may modulate *WNT2* expression.

The linkage disequilibrium with the *DLD* rs134692583:A>T and the potential protective effect of the T allele also at this locus might suggest a combined mechanism of action of these neighbor genes in the resistance to MAP infection. However, functional assays are necessary to clarify if these two SNP modulate binding of transcription factors and thereby regulate their target gene expression and MAP infection susceptibility. The protective role of the haplotype TT could be a useful resource to support and verify the situation of the available 50K SNP Chip data in Holsteins and eventually be implemented in genome wide breeding programmes.

No association with MAP infection was found for the other investigated genes, thus not confirming the results of previous reports (table 1). Further investigation is also required to clarify the possible biological role of these genes in the pathogenesis of Johne's disease.

This study contributes to the understanding of genetic variability involved in Johne's disease susceptibility and it clarifies the involvement of the investigated loci in MAP infection. The identification of loci associated with MAP susceptibility is the first step to set up marker assisted selection programs in order to make cattle populations more resistant, reduce the transmission of MAP to other animals in the herd, improve the health status by breeding and increase the productivity of the livestock industry.

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Table 1 - Positional candidate genes recently indicated as putatively involved in Johne's disease after GWA studies.

SNP ID	BTA	RefSeq genes (1 Mb)	Reference
ss86341066:A>C	3	*EDN2*	Settles *et al.* 2009
rs43070062:C>G	9	-	Settles *et al.* 2009
ss61491930:G>A	7	*IL4, IL5, IL13, IRF1*	Pant *et al.* 2010
ss86328445:T>C	11	*SOCS5*	Pant *et al.* 2010
ARS-BFGL-NGS-8531:A>G	9	*PRDM1*	Minozzi *et al.* 2010
UA-IFASA-8974:A>C	20	*PTGER4*	Kirkpatrick *et al.* 2011
rs41748405:A>C	15	*GNA12*	Zanella *et al.* 2011
ss66537488:C>T	4	*DLD, LAMB 1, WNT2*	van Hulzen *et al.* 2012

Table 2 - Reference SNP chosen for a confirmation study of association with MAP susceptibility. Chromosome, gene location, alleles and amino acid changes are reported. Positions of the SNP related to the gene (5' flanking region/exon/intron) and related to BTA UMD 3.1 are also indicated. Primer sequences, amplicon size (bp) and restriction endonuclease information were provided for PCR amplification and RFLP genotyping. Asterisks indicate polymorphic alleles confirmed in German Holstein.

Gene	BTA	RefSNP	Alleles	Aa change	Gene Pos./ UMD3.1	Primer	Size (bp)	Genotyping
LAMB 1	4	rs43388824	A>G	-	Exon 8 49318278	5'-GGGAAGTAAACTTTACATAAG-3' 5'-GCACGTACTTACCATTC-3'	345	*BsmAI*
LAMB 1	4	rs211391398	C>T*	Ser>Asn	Exon 11 49312998	5'-TTGGTTAAAGATAAAATGAAGC-3' 5'-TTTTGTGAAATTTGGAGGG-3'	402	*DraI*
DLD	4	rs134692583	A>T*	-	5' flank. reg. 50639460	5'-TTACGCTCTTTACGACAGT-3' 5'-TTCTGCCAAGGATTTCAC-3'	168	*SphI*
WNT2	4	rs43390642	G>T*	-	5' flank. reg. 51397892	5'-GGGTGGATGAAATGATGGCAA-3' 5'-TCTACCCCGAGCGCTTG-3'	167	*HaeIII*
PRDM1	9	rs136669229	A>C*	Phe>Val	Exon 2 44375813	5'-CAGAGTCATATCCGCGTC-3' 5'-CGGGACAATGGGGATTAA-3'	103	*NlaIV*
PRDM1	9	rs136770416	A>C	Val>Gly	Exon 5 44359367	5'-TTGATGAGATTCACCGCCT-3' 5'-CTGAAGGACAAGGCCTG-3'	140	*DraIII*
SOCS5	11	rs134378401	A>C	Tyr>Asp	Exon 2 29050621	5'-AGAGCGACTTCCTACAGT-3' 5'-AGTTCACTGGATACGGATAAAA-3'	129	*RsaI*
PTGER4	20	rs41944920	C>G	Leu>Val	Exon 3 33764044	5'-TGATAAGTTCAGCGTTTCAC-3' 5'-AGCCATAGAGAAGATCAAAT-3'	277	*HinfI*

IL10	16	present work	A>G*	-	Intron 4	5'-CATGACCTTCCCAGCAG-3'	353	*Alw26I*
						5'-AATAAATATATGTGGGAGCTGAG-3'		

Table 3. Analysis of transcription factor binding sites in the DNA sequences surrounding the SNP: a) *DLD* rs134692583:A>T; b) *WNT2* rs43390642:G>T and c) *IL10* AC_000173:g.3625A>G (present work) by TFSEARCH software.

a) rs134692583:A>T (8 bp upstream *DLD*) 5'-AGGCCGCGCTCGTGC*[A/T]*TGCGCAGGGCGGGGA-3'

Transcription factor	Consensus sequence*	Position relative to SNP (5'>3')	DNA strand	Binding allele score	
				T	A
USF	NCACG**T**GN	-5 to +2	3'>5'	78.4	78.4
c-Myc	NANCACG**T**GNNW	-7 to +4	3'>5'	76.8	-
C/EBPb	NKN**T**TGCNYAAYNN	-3 to +10	5'>3'	76.0	-
Arnt	NDDNNCACG**T**GNNNNN	-8 to +5	3'>5'	75.9	-
N-Myc	NNC**C**ACGTGNNN	-10 to +1	5'>3'	76.0	77.0
GATA-1	SNNGA**T**NNNN	-5 to +4	5'>3'	75.5	-

b) rs43390642:G>T (164 bp upstream *WNT2*) 5'-AAACACCTCCGTGTG*[G/T]*CCTCGAGCACCCGCG-3'

Transcription factor	Consensus sequence*	Position relative to SNP (5'>3')	DNA strand	Binding allele score	
				G	T
AML-1a	TGC**G**GT	-4 to +1	5'>3'	83.7	-
ZID	N**G**GCTCY**A**TCAYC	-1 to +11	5'>3'	78.9	-
GR	NNNNNNCNNTNTG**T**NCTNN	-13 to +5	5'>3'	-	78.2
c-Ets-1	NC**M**GGA**W**GYN	-9 to +2	3'>5'	75.1	75.1

c) AC_000173:g.3625A>G (14 bp upstream the exon 5 of *IL10*) 5'-CACTGAACACGTCTT*[A/G]*TCTCCCCACACAGCT

Transcription factor	Consensus sequence*	Position relative to SNP (5'>3')	DNA strand	Binding allele score	
				A	G
GATA-X	NGAT**A**AGNMNN	-4 to +6	3'>5'	93.1	-
GATA-1	NNC**W**GAT**A**RNNNN	-3 to +9	3'>5'	90.3	-
SREBP	K**A**TCACCCCAC	-1 to +9	5'>3'	90.1	85.9
GATA-3	NNGAT**A**RNG	-5 to +3	3'>5'	89.1	-

*Nucleotides in the genomic sequences according to the consensus sequences are underlined and the polymorphic nucleotide is marked in bold. Exonic regions are double underlined. Binding score threshold for each allele was set to ≥ 75.0. Nucleotide codes: K = G or T; M = A or C; S = C or G; W = A or T; Y = C or T; R = A or G; D = A, G or T; N = A, G, C or T.

Table 4 - Genotyping data, allele frequency and Hardy-Weinberg equilibrium (p≤0.05) of the SNP chosen as molecular marker for a confirmation study of association with MAP susceptibility in German Holstein population. All Chi square tests have one degree of freedom.

Locus	SNP	Observed genotypes					Allele frequency	
			CC	CT	TT	tot	C	T
LAMB1	<u>rs211391398</u>:C>T	Obs.	231	66	5	302	0.87	0.13
		Exp.	230.78	66.44	4.78			
		χ^2=0.013						
			AA	AT	TT	tot	A	T
DLD	<u>rs134692583</u>:A>T	Obs.	240	54	5	299	0.89	0.11
		Exp.	238.42	57.15	3.42			
		χ^2=0.908						
			GG	GT	TT	tot	G	T
WNT2	<u>rs43390642</u>:G>T	Obs.	287	36	1	324	0.94	0.06
		Exp.	287.11	35.77	1.11			
		χ^2=0.013						
			AA	AC	CC	tot	A	C
PRDM1	<u>rs136669229</u>:A>C	Obs.	5	68	243	316	0.12	0.88
		Exp.	4.81	68.37	242.81			
		χ^2=0.009						
			AA	AG	GG	tot	A	G
IL10	<u>AC_000173</u>:g.3625A>G	Obs.	2	39	282	323	0.07	0.93
		Exp.	1.43	40.14	281.43			
		χ^2=0.259						

Table 5 – Frequencies observed for faecal and ELISA tests for each investigated SNP. Differences between the allele frequencies of the different polymorphisms were calculated by Fisher's exact test using three different phenotypes: (1) fecal positive/negative, (2) ELISA positive/negative, and (3) fecal or ELISA positive/negative. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated for each minor allele (MAF) according to the same scheme. Asterisks show significant value (p≤0.05).

| Test		*LAMB1* rs211391398:C>T				P Fisher	Alleles		OR [95% CI]	P ORs
		CC	CT	TT	tot		C	T	MAF	
(1)	+	116	29	3	148	0.642	261	35	0.87 [0.53-1.41]	0.581
	-	115	37	2	154		267	41		
(2)	+	71	25	2	98	0.510	167	29	1.33 [0.81-2.19]	0.256
	-	160	41	3	204		361	47		
(3)	+	124	37	3	164	0.926	285	43	1.11 [0.68-1.80]	0.670
	-	107	29	2	138		243	33		
		DLD rs134692583:A>T								
		AA	AT	TT	tot		A	T		
(1)	+	126	22	2	150	0.235	274	26	0.64 [0.38-1.09]	0.108
	-	114	32	3	149		260	38		
(2)	+	85	15	0	100	0.173	185	15	0.57 [0.31-1.05]	0.075
	-	155	39	5	199		349	49		
(3)	+	140	24	2	166	0.135	304	28	0.58 [0.34-0.99]	0.046*
	-	100	30	3	133		230	36		
		WNT2 rs43390642:G>T								
		GG	GT	TT	tot		G	T		
(1)	+	149	12	1	162	0.050*	310	14	0.56 [0.28-1.11]	0.098
	-	138	24	0	162		300	24		
(2)	+	103	7	1	111	0.050*	213	7	0.42 [0.18-0.97]	0.042*
	-	184	29	0	213		397	31		
(3)	+	166	13	1	180	0.019*	345	15	0.50 [0.25-0.97]	0.043*
	-	121	23	0	144		265	23		
		PRDM1 rs136669229:A>C								
		AA	AC	CC	tot		A	C		
(1)	+	3	30	124	157	0.583	36	278	0.85 [0.52-1.36]	0.505
	-	2	38	119	159		42	276		
(2)	+	2	26	77	105	0.543	30	180	1.29 [0.79-2.11]	0.295
	-	3	42	166	211		48	374		
(3)	+	3	36	134	173	0.926	42	304	0.95 [0.59-1.54]	0.864
	-	2	32	109	143		36	250		
		IL10 AC_000173:g.3625A>G								
		AA	AG	GG	tot		A	G		
(1)	+	0	22	140	162	0.495	22	302	1.15 [0.61-2.17]	0.657
	-	2	17	142	161		19	301		
(2)	+	0	15	94	109	0.589	15	203	1.13 [0.58-2.19]	0.702
	-	2	24	188	214		26	400		
(3)	+	0	24	155	179	0.493	24	334	1.13 [0.59-2.16]	0.694
	-	2	15	127	144		17	269		
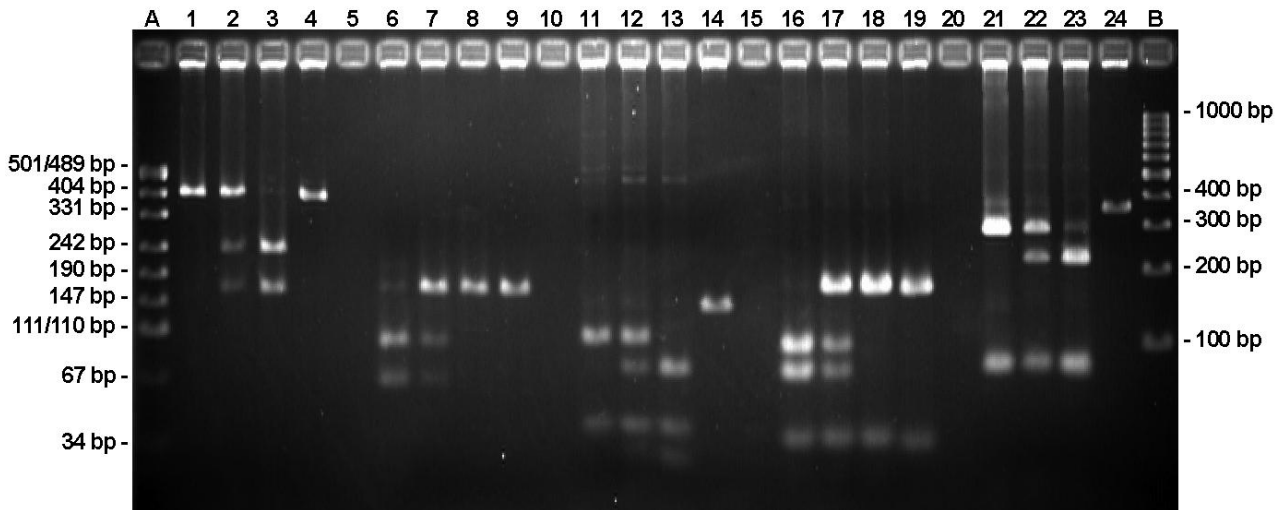
Figure 1. Genotyping of *LAMB1, DLD, WNT2, PRDM1,* and *IL10* by PCR-RFLP in a German Holstein cattle population. Lines 1-4: *locus LAMB1* rs211391398:C>T. Genotypes CC; CT and TT respectively in the lines 1, 2 and 3. Lines 6-9: *locus DLD* rs134692583:A>T. AA, AT and TT are the genotypes reported in the lines 6, 7 and 8 respectively. Lines 11-14: *locus PRDM1* rs136669229:A>C. Genotypes AA, AC and CC respectively reported in the lines 11, 12 and 13. Lines 16-19: *locus WNT2* rs43390642:G>T. GG, GT and TT are the genotypes reported in the lines 16, 17 and 18 respectively. Lines 21-24: *locus IL10* g.3625A>G. Genotypes AA, AG and GG reported in the lines 21, 22 and 23 respectively. Lines 4, 9, 14, 19 and 24 are undigested PCR products each belonging to the relative *locus*. Lines 5, 10, 15 and 20 are empty. Line A is pUC 19 DNA/*MspI* (*HpaII*) Marker, 23 (Fermentas); Line B is GeneRuler™ 100bp DNA Ladder (Thermo Scientific).