Polymorphisms associated to bovine paratuberculosis: investigation of their role in DNA-protein interactions and transcriptional regulation

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Summary
Previous studies led to identify SNPs in putative regulatory regions of the *SLC11A1* and *CARD15* genes with association to paratuberculosis in cattle. Aim of this study was to investigate the role of these mutations at the regulatory level by DNA-protein interaction analyses and transcriptome comparison between wild-type and mutated animals. Gene regions carrying the SNPs of interest were analysed by bioinformatic tools to predict allele-dependent binding sites for transcription factors (TFBS). Putative TFBS were *in vitro* explored by Electrophoretic Mobility Shift Assays (EMSA). EMSA did not show specific gel shifts for any allele indicating that these SNPs may eventually influence gene transcription without altering TFBS. Whole transcriptome expression analysis was performed on intestinal tissues of wild-type and mutated cattle by RNA-Seq. Differential regulation of five genes involved in innate immune system was detected. Specifically, *ULBP3* was down-regulated, while *S100A8, S100A12, LOC510860*, and *IFI27* were up-regulated. In previous studies, *ULBP3*, *S100A8*, and *S100A12* resulted differentially expressed in cattle affected by paratuberculosis, suggesting a possible implication in the pathogen response. Further investigations are needed to elucidate the functional role of these SNPs and to understand the gene network involved in the interactions between non-coding SNPs and other genome regions.

Introduction

Paratuberculosis, or Johne's disease, is a chronic enteritis of ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is one of the most important diseases of dairy and beef cattle, resulting in large economic losses. Some Authors suggested a possible role of MAP in the inflammation observed in Crohn's disease, an inflammatory bowel disease of humans (Olsen *et al.* 2009) although the causal role of MAP has not been proven yet (Mendoza *et al.* 2009, McNees *et al.* 2015). Prevalence around the world was estimated from 1.5 to 70% (Eltholth *et al.* 2009). In a recent survey of 48 countries paratuberculosis was found to be very common in livestock. In about half the countries more than 20% of herds and flocks were infected with MAP (Whittington *et al.* 2019). In Italy, prevalence has been reported to be around 20-40% (Pozzato *et al.* 2011). In Kazakhstan, one seropositive animal to paratuberculosis was detected in saiga antelope (*Saiga tatarica tatarica*) (Orynbayev *et al.* 2016), but the significance of this finding is uncertain. However, MAP infection was also detected in cattle imported from abroad in East Kazakhstan region (Aida Daugaliyeva, unpublished data).

An association between host genetics and paratuberculosis was identified by previous case-control studies based on the candidate gene approach. Single nucleotide polymorphisms (SNPs) represent common mutations that may modulate the response to pathogen exposure. Associated SNPs often occur in open reading frame, thus acting by changing the amino acid composition of the related protein, but they can also be located in non-coding regions (e.g., gene promoter, introns). The latter may act by a plethora of functional mechanisms, including direct alteration of transcription factor binding sites (TFBS) or gene transcriptional modifications, which lead to differential host response following exposure to pathogens.

A strong genetic involvement with susceptibility to paratuberculosis was established for Solute carrier family 11 member 1 (*SLC11A1*) gene in mouse models (Roupie *et al.* 2012). Moreover, polymorphisms associated with susceptibility were reported also in sheep and goats (Reddacliff *et al.* 2005, Korou *et al.* 2010). *SLC11A1* variants in cattle were first described by Pinedo and colleagues (Pinedo *et al.* 2009) in Holstein, Jersey and Brahman-Angus cross breeds. Ruiz-Larrañaga and colleagues (Ruiz-Larrañaga *et al.* 2010) carried out a SNP-based candidate gene study on Holstein-Friesian cattle.

Caspase recruitment domain 15 (*CARD15*) was described as the first susceptibility gene for Crohn's disease in humans (Hugot *et al.* 2001, Ogura *et al.* 2001). In cattle Pinedo and colleagues (Pinedo *et al.* 2009) screened adult cows for SNPs reported in humans, suggesting a role for *CARD15* also in the susceptibility of cattle to Johne's disease.

This study aimed to get further insight on the functional role of three SNPs of *SLC11A1* (c.-90 A > C in the putative promoter; c.1157-91 A > T in intron 11) and *CARD15* (c.2886-14 A > G in intron 10) genes, previously associated to resistance/susceptibility to bovine paratuberculosis, by investigating DNA/protein interactions and by comparing the transcriptomes of wild-type and mutated animals.

Materials and methods

Sampling was carried out from 8 Friesian cattle with more than 2 years of age directly at the slaughterhouse. Small intestine was sampled and an aliquot was washed with sterile water and immediately placed in RNAlater. Another aliquot of intestinal tissue from each animal was used for genotyping of the selected genetic polymorphisms.

DNA was extracted from 50 mg of tissue by the Gentra Puregene Tissue kit (Qiagen) and quantified with the Qubit 3.0 fluorimeter (Thermo Fisher). PCRs were prepared in 50 µl of final volume containing 1X buffer, 2 mM of $ClMg_2$, 1 U of Platinum Taq (Invitrogen), 0.2 mM of dNTPs and 300 nM of each primer. For the genotyping of the polymorphisms of interest, the following primer pairs were used: c.1157-91 A > T in intron 11 of *SLC11A1* gene, primers N1_9_F (5'-TTCAGCAATCAGCTGTAGGG-3') and N1_9_R (5'-GCATCAGTTTTTCCATCTGC-3'); c.-90 C > A in the putative *SLC11A1* promoter, primers N1_2_F (5'-CCAGTGCCTCTTTCTTCTGG-3') and N1_2_R (5'-TCTGGATTGTGGTTGAGATCC-3'); c.2886-14 A > G in intron 10 of *CARD15* gene, CARD15_11_F (5'-GAATTCATTGGGAATCTCAGACAG-3') and CARD15_11_R (5'-CAGGACTAGAGGTCTGAG CCATAA-3').

Target sequences were analysed by MATCH (http://www.gene-regulation.com/pub/programs.html#match), PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), TFIND (http://tfbind.hgc.jp/), and Jaspar (http://jaspar.genereg.net/) software for the prediction of putative TFBS. Putative TFBS to be investigated were selected when all the software provided consensus outputs.

Sequence regions flanking the polymorphisms of interest were selected to design double-strand hybridization probes according the protocol described by Holden and Tacon (Holden and Tacon 2011). NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher) were used to obtain nuclear extract from bovine intestine. ElectroMobility Shift Assays (EMSA) experiments were carried out using the LightShift Chemiluminescent EMSA kit (Thermo Fisher) on both alleles of each SNP following the manufacturer's protocol.

Total RNA was isolated from the 8 bovine intestine samples using the RiboPure RNA Purification kit (Agilent Technologies). RNA-Seq libraries were prepared employing the TrueSeq RNA Library Prep (Illumina) and analysed on a Illumina HiSeq platform. Sequence data were compared by Principal Component Analysis (PCA) and Clustering Analysis.

Results

The genotyping of the three polymorphic sites under study showed the presence of wild-type and mutated alleles in the analysed cattle. Specifically, for the c.-90 A > C polymorphism in the *SLC11A1* promoter, the A/C genotype was found in three animals. As regards the c.1157-91 A > T polymorphism in intron 11 of the *SLC11A1* gene, the A/T genotype was found in two cases. Finally, for the polymorphism c.2886-14 A > G of intron 10 of *CARD15* gene, one animal carried the A/G genotype.

The analysis of polymorphic and wild-type regions, carried out in parallel using different software, allowed to identify three putative TFBSs. For the *SLC11A1* gene, software predictions showed that the sequence of the promoter containing cytosine create a recognition site for the Sp1 transcription factor, which instead would not be able to bind to DNA in the presence of adenine. As regards the polymorphism localized in intron 11 of the same gene, bioinformatics predicted a recognition site of the transcription factor MYOD for the sequence containing thymine. Also for *CARD15*, it could be identified a binding site for GATA3, which can no longer be recognized when guanine, associated with the disease susceptibility, replaces adenine. These predictions were *in vitro* investigated by setting up EMSA experiments. EMSA is expected to display gel shifts in case of reaction between the putative binding sites and transcription factors contained in the nuclear protein extract (Hellman and Fried 2007). However, EMSA assays carried out to investigate possible interactions between *SLC11A1* c.-90/Sp1 and *CARD15*/GATA3 did not show any shift, thus not confirming the hypotheses raised *in silico*. The analysis of the binding between *SLC11A1* c.1157-91 and MYOD instead showed a gel shift in the presence of the probe carrying adenine (Figure 1).

With regard to the comparison of transcriptomes of wild-type and mutated cattle, the optimization of tissue collection (i.e. stored in RNAlater directly at the slaughterhouse) and extraction procedure allowed to obtain RNA of good quality from intestine tissue. RNA Integrity Number (RIN) values of the samples ranged between 6.6 and 7.9, with the exception of one sample that had a RIN of 5.2 and it was therefore excluded from the analyses. RNA-Seq analysis revealed high variability, as demonstrated by PCA and Cluster analyses (Figures 2 and 3), which show that samples group independently from the mutations. As a consequence, we observed non significant modulations of the expression of several genes. This was not completely unexpected due to the limited number of animals enrolled in the study. Nevertheless, a panel of genes were significantly modulated according to *SLC11A1* genotype, and they were: UL-16 binding protein 3 (*ULBP3*), which was down-regulated [$\log_2$(FC) = - 3.81] in the status g.-90 A > C; calcium-binding protein S100A8 and S100A12 (*S100A8, S100A12*), c4b-binding protein alpha-like (*LOC510860*) and interferon α inducible

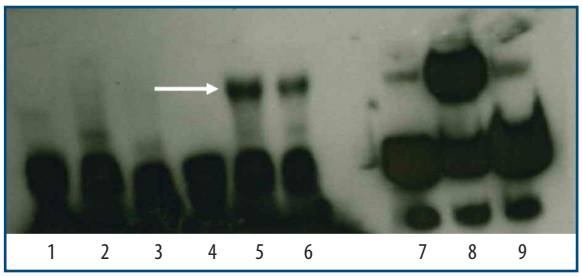

Figure 1. *Electrophoretic mobility shift assay (EMSA) for SNP g.1057-91 in intron 11 of* SLC11A1. NE = nuclear extract from bovine intestine. The arrow indicates the detected gel shift.
SNP: 1 = SLC11A1 g.1057-91_T_bio;
2 = SLC11A1 g.1057-91_T_bio + NE;
3 = SLC11A1 g.1057-91_T_bio + NE + competitor;
4 = SLC11A1 g.1057-91_A_bio;
5 = SLC11A1 g.1057-91_A_bio + NE;
6 = SLC11A1 g.1057-91_A_bio + NE + competitor;
CTRL: 7 = EBNA DNA bio;
8 = Ctrl EBNA DNA bio + EBNA NE;
9 = EBNA DNA bio + EBNA NE + competitor.

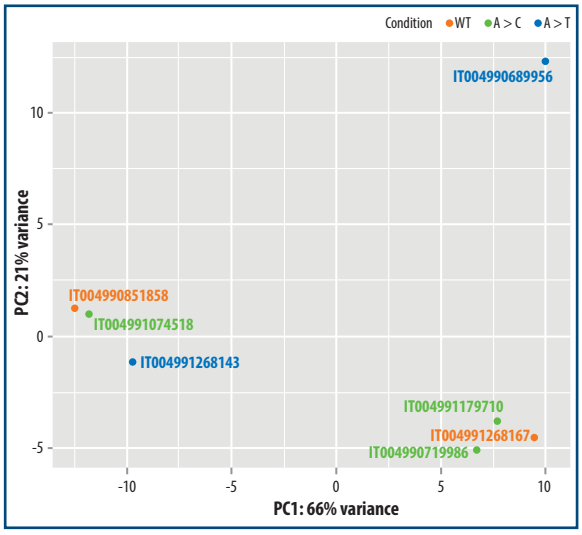

Figure 2. *Principal Component Analysis on data from bovine intestine samples analyzed by RNA-Seq.* Wild-type animals are in orange; mutants for SNP g.-90 are in green; mutants for SNP g.1157-91 are in blue.

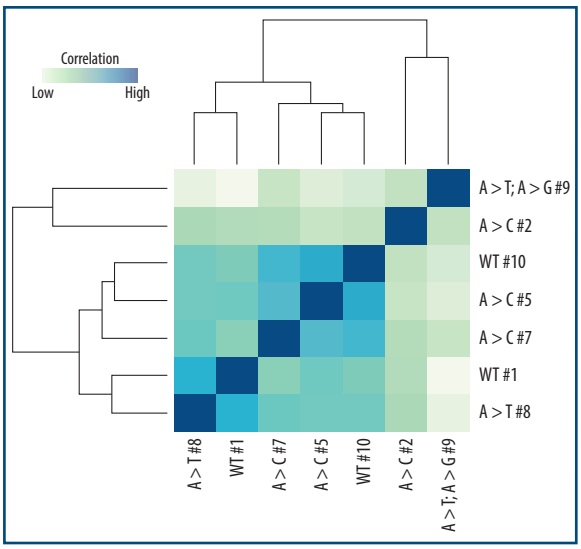

Figure 3. *Heatmap of sample-to-sample distances for bovine intestine samples analyzed by RNA-Seq.* WT = wild type animal; A > T = mutant for SNP g.1157-91; A > C = mutant for SNP g.-90.

protein 27 (*IFI27*), which were up-regulated in the status 1157 g.-91 A > T compared to the wild-type genotype, with $\log_2$(FC) of 6.86, 6.63, 3.70 and 4.34, respectively.

Discussion

Bioinformatic analysis allowed the identification of three putative TFBS whose consensus sequence was predicted as conserved or disrupted depending on SNP allele. With regard to *SLC11A1* gene, SNPs in the promoter and in the intron might constitute sites for Sp1 and for MYOD, while a TFBS for GATA3 was predicted for *CARD15* intronic sequence. However, EMSA experiments did not show specific gel shifts, meaning that there are not altered TFBS in correspondence of the polymorphisms of interest. The only exception was a gel shift for *SLC11A1* c.1157-91 in the presence of the probe carrying adenine, and not with the wild-type allele (thymine) as predicted *in silico*. This result suggests that likely another transcription factor than MYOD exists, capable of recognizing intron 11 of *SLC11A1*.

Actually, studies conducted on the promoter activities showed that polymorphisms located within 500 bp upstream the transcription start site (as the g.-90 C > A in the *SLC11A1* putative promoter) can influence the transcription levels and concur positively to the promoter activity (Chorley *et al.* 2008, Hudson 2003). Moreover, non-coding polymorphisms might have a role on flexibility of DNA strand and chromatin folding (Elkon and Agami 2017). Therefore, the SNPs of interest could have an indirect influence in the regulation of gene expression and further studies are needed to elucidate their functional role.

RNA-seq analysis allowed to identify a significant expression difference for five genes even though the variability in the samples was quite high, likely because the animals have been compared based on SNP allele, which does not determine a sharp separation condition (i.e. healthy/diseased). Noteworthy, these genes are involved in the innate immune response and three of them (*ULBP3, S100A8* and *S100A12*) were already shown to be differentially expressed in cattle affected by paratuberculosis. Shin and colleagues (Shin *et al.* 2015) carried out a hybridization study on microarray using RNA obtained from whole blood of healthy and paratuberculosis-affected cattle, and they found *ULBP3* to be down-regulated in cattle affected by the disease. We observed the same expression pattern for *ULBP3* gene in *SLC11A1*-mutated cattle: however, it should be taken into account that the mutated A allele in the *SLC11A1* promoter was previously associated with disease resistance. The *ULBP3* gene encodes for a GPI-anchored protein and acts as a ligand for the NKG2D receptor of NK cells. When the receptor is activated following the binding with the ligand, the NK cell is activated for the recognition of invasive cells (Kasahara *et al.* 2012, Larson *et al.* 2006, Mou *et al.* 2014).

Verschoor and colleagues (Verschoor *et al.* 2010) conducted microarray and RT-qPCR analyses on whole-blood RNA from healthy and MAP-infected cattle showing different expression levels for the *S100A12* and *S100A8* genes. Holstein cattle showed up-regulation of these genes, as found in our animals, which were Friesian cattle, a breed related to Holstein. *S100A8* encodes a calcium-binding protein involved in innate immunity reactions and antimicrobial activities. The protein is localized in the cytosol of neutrophils, monocytes, leukocytes, fibroblasts, tumor cells, bone marrow (Donato *et al.* 2013). In humans, elevated protein levels have been reported in cases of rheumatoid arthritis and inflammatory bowel disease, as well as in cases of bronchitis and tuberculosis, suggesting that this protein may play a role in the inflammation reaction (Ikhtaire *et al.* 2016). In particular, S100A8 protein plays a role in the migration of neutrophils to inflammation sites (Vandal *et al.* 2003). In addition, Park et al.and colleagues (Park *et al.* 2016) conducted a study to find marker genes for the detection of MAP infections. The authors compared RNA extracted from whole blood of healthy cattle and cattle affected by paratuberculosis by RT-qPCR and they showed an up-regulation of the gene in diseased animals. *S100A12* encodes a calcium-binding protein with phagocytic and pro-inflammatory activity (Donato *et al.* 2013). This protein is a ligand for RAGE and the RAGE-S100A12 complex forms a pro-inflammatory axis that participates in many inflammatory diseases and has a central role in innate immunity (Chen *et al.* 2010).

The *LOC510860* gene encodes an alpha-like c4b-binding protein that has also been identified in the bovine genome. In humans, c4b-bindin protein is able to inhibit the activation of the classic pathway of the complement system. Moreover, it has been shown that this protein is able to bind to the hypervariable region of *Streptococcus pyogenes* thus protecting the bacterium from complement-mediated opsonization (Areschoug *et al.* 2004).

The *IFI27* gene codes for an interferon alpha inducible protein 27. *IFI27* induces apoptosis by sending extracellular signals to activate other pro-apoptotic genes. It is a membrane protein induced by interferon alpha and beta. In humans, it is up-regulated in the blood of men with prostate cancer undergoing radiation therapy (Hsiao *et al.* 2013).

In conclusion, this study provided further insights into the role of SNPs associated with resistance/

susceptibility to bovine paratuberculosis. The studied SNPs did not show direct effects on the mechanisms of regulation of DNA-protein interactions. RNA-seq analysis allowed to identify a panel of genes differentially expressed depending on SNP alleles; most of these genes exert their action in immune defense pathways. These results deserve further investigations in order to understand direct and indirect (gene network) mechanisms by which non-coding *SLC11A1* SNPs interact with other loci in the genome. Whole genome scan analysis might help to identify other genetic factors associated with the investigated polymorphisms.

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