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Evidence for gene-gene epistatic interactions between susceptibility genes for *Mycobacterium avium* subsp. paratuberculosis infection in cattle

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ABSTRACT

Johne's disease is a chronic granulomatous inflammatory disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), with a particularly negative impact on the economy of the dairy industry. In recent years, several whole genome and candidate gene association studies have been published reporting MAP susceptibility genes, but the putative interaction between them remains unknown. Here, twenty-four single nucleotide polymorphisms in the bovine *SLC11A1*, *NOD2*, *SP110*, *TLR2*, *TLR4*, and *CD209* genes, previously related with paratuberculosis disease, have been analyzed. Several significant ($P < 0.05$) pair-wise genetic interactions were detected: *CD209-TLR4*, *CD209-TLR2*, *TLR4-TLR2*, *SP110-SLC11A1*, *SP110-TLR2*, and *SP110-NOD2*. The statistical interaction described here between bovine *CD209* and *TLR4* genes may be indicative of the biological interaction between their protein products upon infection by mycobacteria, as has been reported to occur in humans. Overall, this is the first evidence of epistasis among bovine innate immunity genes affecting susceptibility to MAP infection, corroborating, in turn, their implication in the disease.

1. Introduction

Paratuberculosis (PTB) is a chronic granulomatous inflammatory disease caused by the infection by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which affects ruminants worldwide. The disease leads to significant economic losses, particularly to the dairy industry, due to reduced milk production, reduced fertility and higher management costs (Lombard, 2011).

Several candidate gene and genome wide association studies (WGAS) have been published during recent years in an effort to identify the susceptibility *loci* explaining the heritability for MAP infection status in cattle (Purdie et al., 2011). The bovine *SLC11A1*, *NOD2*, *SP110*, *TLR2* and *TLR4* genes have been described by our research team as MAP susceptibility *loci* in Spanish Holstein-Friesian (HF) cattle based on case-control association studies (Ruiz-Larrañaga et al., 2010a, 2010b, 2010c, 2011). In a more recent association study, these five genes have been analyzed, together with *CD209* (*CD209 molecule*), in the context of histopathological forms of the disease where "cases" were defined based on the presence or not of lesions in their tissues; among the six *loci*, only *CD209* seems to be implicated in the development of lesions related with the latent form (Vázquez et al., 2014a).

It is well recognised that gene-gene interactions, also known as epistasis, could be one of the potential mechanisms implicated in complex diseases, together with gene-environment interactions (Cordell, 2002; Millstein et al., 2006). Indeed, the existence of interactions between *loci* has been pointed out also as a main reason for the lack of success in genetic association studies of complex diseases (Moore, 2003). Several studies have pointed out the relevance of this phenomenon in the susceptibility to human complex diseases (Hughes et al., 2012; Kumar et al., 2012; Maurya et al., 2014).

Among the existing approaches directed to the identification of such interactions, the "case-only" epistasis analysis focused on affected individuals has been shown to be a more powerful test for epistasis compared to case-control analysis, since it results in greater precision (i.e., smaller standard errors) when estimating interactions (Yang et al., 1999; Cordell, 2009; Maurya et al., 2014). "Case-only" analysis exploits the fact that an interaction term in the logistic regression equation corresponds to the dependency or the correlation between the relevant predictor variables within the population of cases, always assuming

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that the variables are not correlated in the general population and fit Hardy-Weinberg equilibrium (HWE) (Cordell, 2009). The "case-only" design has been used extensively for the assessment of gene–environment and gene–gene interactions (Lupo et al., 2014; Maurya et al., 2014; Afshar et al., 2016). Overall, the general objective of the present study is to explore the putative gene-gene interactions between *SLC11A1*, *NOD2*, *SP110*, *TLR2, TLR4* and *CD209* genes in the context of susceptibility to MAP infection by a case-only epistasis analysis.

2. Materials and methods

2.1. Sampling and phenotype definition

Blood and tissue samples from 780 animals slaughtered at two local abattoirs in the Basque Country (Northern Spain) were collected. A systematic sampling was weekly performed from March 2007 to May 2010. In each sampling day, the average number of animals selected for the study varied from 4 to 6. Animals were chosen according to breed (only Holstein-Friesian cattle) and age requirements (preferably aged 30–60 moths), following the slaughter line order fixed by the slaughterhouse managers. Adult cattle were chosen because the chances of being exposed to MAP were higher than those for younger animals.

Immediately after stunning and before bleeding, duplicate jugular venous whole blood samples were collected into 10 mL Vacutainer EDTA tubes (BD, Franklin Lakes, USA) to later perform the immunological and SNPs genotyping processes. Then, the gut package of each animal was identified and picked up, macroscopic examination was performed and tissue samples for subsequent microbiological and histopathological studies were selected. Further details regarding sampling procedure have been published elsewhere (Vázquez et al., 2014a).

Operations in both municipally owned companies were authorized by slaughterhouse management and carried out under the supervision of official veterinarians and complied with the pertinent legislations for safeguarding animal welfare (Basque Government Decree 454/1994, Spanish Government Law 32/2007 and Royal decree 731/2007, and European Council Regulation (EC) Number 1099/2009). The date of birth of the animals, recorded in the EU bovine identification documents (Council Regulation (EC) Number 1760/2000), were provided by the slaughterhouse veterinary inspectors.

The status of MAP infection was characterized by serological, microbiological, and histopathological methods, in order to include all currently known immunopathological forms of bovine paratuberculosis, as described in Vázquez et al. (2014b). Briefly, the humoral response was evaluated by a two-step indirect Pourquier ELISA paratuberculosis kit (Institut Pourquier, Montpellier, France), currently IDEXX Paratuberculosis Screening Ab Test, and IDEXX Paratuberculosis Verification Ab Test; (IDEXX Laboratories, Inc., Westbrook, Maine, US). Then, MAP was isolated from two homogenates of three tissue samples [ileocecal valve (ICV) and distal ileum (DI) in one, and jejunal caudal lymph node (JC-LN) in the other] by duplicate culture in Herrold (Becton & Dickinson, MD, US) and Löwestein-Jensen (Difco, Detroit, Michigan, US) media, supplemented with mycobactin J (Allied Monitor, Fayette, Missouri, US), as described by Juste et al. (1991). Simultaneously, a second aliquot of the same homogenate was processed for the identification of MAP by using a combined Adiapure®-Adiavet® DNA extraction, purification and amplification assay for MAP specific IS*900* (Adiagene, Saint Brieuc, France), as reported elsewhere (Vázquez et al., 2013). Finally, histopathological examination was performed on formalin-fixed ICV, DI, JC-LN, and ileal LN sections routinely processed and stained with hematoxylin and eosin, and lesions were classified according to González et al. (2005) and Vázquez et al. (2013).

Following previous criteria (Ruiz-Larrañaga et al., 2010a, 2010b, 2010c, 2011), a case (infected animal) was defined as an individual who was positive for any of the tests and a control (healthy animal) was defined as an individual who was negative for all the tests. As a result, the final population consisted of 491 cases, and 289 controls. Among cases, the percentage of animals positive for each test was: ELISA (15%), PCR (53%), culture (30%) and histopathology (76%) (Supplementary Material, Table S1). The use of four different diagnostic tests allowed us to ensure a low percentage of false positive and false negative animals.

Table 1
Single nucleotide polymorphisms analyzed in the bovine *SLC11A1, NOD2, SP110, TLR2, TLR4* and *CD209* genes.

Gene	Chromosome (BTA)	SNP	Association *P*-value[a]	Reference of *P*-value
SLC11A1	2	rs109453173	0.037	Ruiz-Larrañaga et al., 2010a
		rs110090506	0.023	
SP110	2	rs136859213	0.013	Ruiz-Larrañaga et al., 2010c
		rs133080973	0.0003[b]	
		rs110480812	0.0003[b]	
CD209	7	rs208222804	0.002	Vázquez et al., 2014a
		rs209491136	0.047[c]	
		rs211654540	0.006[c]	
		rs208814257	0.015[c]	
		rs210748127	0.009[c]	
TLR4	8	rs29017188	< 0.008[c]	Ruiz-Larrañaga et al., 2011
		rs43578097	< 0.008[c]	
		rs43578100	< 0.008[c]	
TLR2	17	rs41830060	0.013[b]	Ruiz-Larrañaga et al., 2011
		rs43706434	0.013[b]	
		rs110491977	0.013[b]	
		rs43706433	0.013[b]	
		rs68268259	0.013[b]	
		rs41830058	0.013[b]	
		rs109971269	0.013[b]	
NOD2	18	rs109601360	0.038[b]	Ruiz-Larrañaga et al., 2010b
		rs43710288	0.038[b]	
		rs43710289	0.038[b]	
		rs43710290	0.017	

[a] Association *P*-values in previous studies of our group.
[b] Haplotypic association.
[c] Genotypic association.

2.2. SNP genotyping

A total of twenty four SNP in six bovine innate immunity system genes were typed for the present study: *SLC11A1* (2), *SP110* (3), *CD209* (5), *TLR4* (3), *TLR2* (7), and *NOD2* (4) (Table 1). All SNP were significantly associated with paratuberculosis in previous results of our research group. The SNPs were genotyped using TaqManOpenArray technology (Life Technologies, Carlsbad, USA), and subsequent allele assignation was carried out using Autocaller v1.1 software (Life Technologies, Carlsbad, USA). Each genotyping array included duplicated negative and positive controls. All SNPs were successfully genotyped (call rate > 80%). One hundred and thirty-six animals (83 cases and 53 controls) were excluded for subsequent analyses because of their low genotyping rate (call rate < 80%).

2.3. Statistical analysis

In order to assess if the application of a "case-only" approach was suitable or not for gene-gene interaction analysis, HWE, linkage disequilibrium and genotypic disequilibrium tests were performed on the whole recruited population of 780 animals using Haploview v.4.2 (Barrett et al., 2005) and GENEPOP v.4.2 (Rousset, 2008).

The following "case-only" pair-wise epistasis analysis was performed using PLINK v.2.050 software (Purcell et al., 2007) in a parametric approach. This method uses a logistic regression test for interaction, which assumes an allelic model for both the main effects and the interactions. Due to the number of analyzed SNP, the false