Short Communication: Association Analysis of Microsatellites and *Mycobacterium avium* Subspecies *paratuberculosis* Antibody Response in German Holsteins

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ABSTRACT

Paratuberculosis in ruminants is characterized by chronic granulomatous enteritis, resulting in persistent diarrhea and progressive wasting of cattle infected with *Mycobacterium avium* ssp. *paratuberculosis* (MAP). The disease occurs worldwide with high frequency, leading to growing economic losses in beef and dairy industries. The objective of this study was to investigate associations of microsatellites (*BMC9006*, *BB704*, *BB705*, *BB717*, *BB719*, *BMS1617*, *BB702*, and *BOBT24*) located near or within candidate genes involved in response mechanisms to paratuberculosis. Pedigree information existed for 4,686 German Holstein cows that had routinely been screened for MAP status using commercially available serum antibody ELISA test. The immunoglobulin G cutoff level was used to classify all animals as positive or negative for paratuberculosis. A total of 594 (12.7%) cows tested positive for paratuberculosis. The control group comprised 585 animals testing negative for MAP. Microsatellite *BMC9006* had only 3 alleles (2 of which occurred at very low frequencies in the present data set) and was therefore not informative; the remaining microsatellites showed 3 to 12 alleles. Fisher's exact and χ^2 tests revealed no significant differences in microsatellite allele frequencies between the 2 groups of German Holstein cows testing positive or negative for paratuberculosis.

Key words: paratuberculosis, microsatellite, susceptibility, German Holstein

Paratuberculosis in ruminants is caused by *Mycobacterium avium* ssp. *paratuberculosis* (MAP). Clinical disease is characterized by a chronic granulomatous enteritis, persistent or intermittent diarrhea, and excessive loss of weight. Worldwide economical losses due to MAP

result from the long incubation period, reduced performance in production traits, and lack of effective therapy (reviewed by Chacon et al., 2004). *Mycobacterium avium* ssp. *paratuberculosis* is potentially a crucial factor in the development of Crohn's disease in humans (Greenstein, 2003; Grant, 2005).

Breeding strategies to enhance resistance of cattle against mycobacterial infections could be an additional method to hygienic programs in combating paratuberculosis. The estimated heritability of paratuberculosis in different goat breeds ranges from 0.01 to 0.15 (Singh et al., 1990). Heritability based on microbiological and histological postmortem examination of slaughtered cows was assessed by Koets et al. (2000). They estimated a heritability of 0.06 for the overall population of vaccinated and nonvaccinated cattle. A Danish study tested milk samples of 11,535 Danish dairy cows using ELISA. When based on a multivariate model, the data showed a heritability of 0.10 for the humoral antibody response. Applying a univariate model with whole pedigrees, a heritability of 0.10 was attained. Implementing a sire model showed a heritability of 0.09. A low and nonsignificant correlation (−0.037) was also found between antibody level and daily milk yield (Mortensen et al., 2004). Gonda et al. (2006a) studied daughters of 12 sires previously tested for paratuberculosis infection by using both ELISA and fecal culture. The combined test resulted in an estimated heritability of 0.10. Gonda et al. (2006b) also identified a QTL for paratuberculosis susceptibility on *Bos taurus* autosome 20 in US Holsteins.

All preceding studies confirm genetic influence on paratuberculosis susceptibility despite low heritability estimates.

Genetic resistance is the inherent capacity of a previously unexposed animal to resist disease when challenged by pathogens (Adams and Templeton, 1998). Although there is no particular gene known to be responsible for differences in paratuberculosis susceptibility in cattle, *SLC11A1* (formerly natural resistance associated macrophage protein 1, *NRAMP1*) is associ-

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ated with natural resistance to infection with *Salmonella* spp., *Leishmania* spp., and *Mycobacterium* spp. in the mouse (Vidal et al., 1993). Genetic variants of *SLC11A1* in humans are partly responsible for resistance against tuberculosis (Bellamy, 2000) and have been associated with resistance against *Brucella abortus* infection in cattle (Adams and Templeton, 1998; Barthel et al., 2001), but do not determine resistance or susceptibility to bovine tuberculosis (Barthel et al., 2000). Reddacliff et al. (2005) discovered a possible association of one *SLC11A1* microsatellite allele with severe clinical paratuberculosis in sheep. Furthermore, IL-12 is a crucial cytokine in control of mycobacterial infections. Mutations in the *IL12* gene and its receptor lead to increased susceptibility to mycobacterial infections (reviewed by Basu, 2004). Tumor necrosis factor- α response to mycobacterial antigens is weaker in the blood of MAP-infected cattle compared with uninfected ones, which may have negative effects on granuloma formation and function (Buza et al., 2003). Expression of the *IL10* gene was increased in tissues of clinically diseased cows compared with healthy controls and subclinically infected animals (Khalifeh and Stabel, 2004). Finally, just as IFN- γ is a major cytokine involved in macrophage activation and resistance to mycobacteria (Stabel, 1996), IL-18 is an important regulator of innate and acquired immune responses (Akira, 2000). The aim of this study was to analyze possible associations of microsatellites located near or within these candidate genes with susceptibility to MAP in cattle.

Eight microsatellites were chosen for this purpose due to their close linkage to the above mentioned candidate genes involved in immune response against mycobacteria (Table 1).

Pedigree information existed for 4,686 German Holstein cows originating from 13 German farms. All of these animals had routinely been screened for MAP status within a surveillance program in Thuringia in spring 2005. Herd sizes varied from 53 to 784 animals. The occurrence of animals testing positive ranged between 2.8 and 36.8% within farms. Complete lactation data were available for 4,590 of these cows. To describe the lactation yield of all tested animals, the mean and standard deviations for milk production traits are presented in Table 2 (records with DIM below 250 d were excluded). Antibody levels were determined by using commercially available ELISA tests (IDEXX test, IDEXX, Wörrstadt, Germany; Svanovir, Svanova, Uppsala, Sweden) and results were recorded as optical density (OD) percentage values or sample-to-positive (S/P) values for each cow, respectively. The test-specific IgG antibody cutoff level (IDEXX: S/P value = 0.25; Svanovir: OD = 53%) was used to classify all animals as positive or negative for paratuberculosis, which re-

sulted in a total of 594 (12.7%) positive cows. The control group comprised 585 animals testing negative for MAP, each of which correlated in age at the time of testing, was descended from the same sire, and originated from the same farm as at least 1 of the positive animals. Regarding both groups, the animals descended from 246 sires with an average number of 4.8 progeny. If no matching half-sib was present in the population, older animals born on the same farm and with repeated negative test results were chosen as controls. The average age of the negative control group was slightly higher (51.3 mo) than the age of the positive animals (48.7 mo), which was expected because of choosing older animals as controls in cases of no matching half-sibs available.

The DNA was extracted from whole blood samples using Invisorb Blood Mini HTS 96-Kit/C-Sheep (Invitex GmbH, Berlin, Germany) and stored at 4°C before use. Details on microsatellites used in this study are shown in Table 1. In each case, a fluorescent dye [FAM (6-carboxyfluorescein), VIC, or NED] was attached to the 5' end of the reverse PCR primer. As nontemplated addition of a nucleotide to the 3' end of the PCR-amplified product of *BB704* and *BMC9006* occurred, the sequence GTTCTT was added to the 5' end of the reverse primer to facilitate accurate genotyping (Brownstein et al., 1996). Microsatellite loci were coamplified using the AbsoluteQPCR Mix (ABgene Germany, Hamburg, Germany) in 2 groups: *BB704*, *BB705*, *BB717*, *BB719*, and *BMC9006* (multiplex 1) and *BMS1617*, *BB702*, and *BOBT24* (multiplex 2). One nontemplate negative control and a reference sample were amplified in every PCR reaction to standardize the results. Reactions were carried out in a total volume of 15.0 μ L, containing 50 ng of target DNA, 1 $\times$ AbsoluteQPCR Mix (ABgene Germany), and 1 $\times$ primer mix. Final concentration for each primer pair varied between 0.5 and 1.25 pmol/ μ L. The PCR reactions were performed in 96-well microtiter plates using an iCycler temperature cycler (Bio-Rad, München, Germany) programmed for 15.0-min denaturation at 95°C, followed by 30 cycles of 0.5-min denaturation at 94°C, 1.5-min annealing at 62°C (multiplex 2) or 63°C (multiplex 1), and 1.0-min extension at 72°C, followed by a final extension period of 30.0 min at 60°C. Fragment analysis was performed using the ABI Prism 377 DNA Sequencer and Genotyper (V 3.7 NT) Gene Scan Analysis software (both from Applied Biosystems, Darmstadt, Germany).

For each microsatellite, allele frequencies between MAP-positive animals and the control group were compared using the χ^2 and Fisher's exact tests. Allele frequencies below 5% were grouped together for this statistical test (see Table 3). Besides *BMC9006*, the microsatellites *BB704*, *BB705*, *BB717*, *BB719*, *BMS1617*,

Table 1. Primer sequences (underlined = PIGtail) of forward (f) and reverse (r) primers, fluorescent labels (FAM, VIC, NED), and range of allele size of microsatellites

Microsatellite	Locus	<i>Bos taurus</i> autosome	Primer sequences (5' → 3')	Allele size (bp)
BB704 ¹	IL12A	1	GTTTCTTTTAAGCAGGGACGAACGTG (f) NED-AATTGCCAGGAAGATTGTGG (r)	107–145
BMC9006 ²	SLC11A1	2	GTTTCTTGTGGAATGAGTGGGCACAG (f) FAM-CCTTCCAGAACTCCCTCTCC (r)	223–231
BMS1617 ³	IFN- γ	5	GCCTGCATGTGTCTGTGG (f) FAM-TCTGTGTCTGGAATACCCTCC (r)	143–165
BOBT24 ⁴	IL4	7	GAGCAAGGGAATTCAGTGGAGC (f) NED-TGTATTTTACATTCAGGTCTGTGATCC (r)	146–165
BB719 ¹	IL12B	7	AAATGCCAGGACCTCACAG (f) VIC-GCTAGGAGATGTTGCTGCTG (r)	254–284
BB702 ¹	IL18	15	TAGCCACTGGACCATCAGG (f) VIC-TGGCATTAGACCATGACAGC (r)	186–198
BB717 ¹	IL10	16	TGAGTCACCTGCAAGTTTGA (f) VIC-GTCTGGTCCCAGTTTTGCA (r)	152–164
BB705 ¹	TNF- α	23	GGGAGGTCCTTTCCTACAGTGC (f) FAM-CACAAAGAGTCAGACACAGTTTAGC (r)	118–128

¹Sonstegard et al. (2000).²Feng et al. (1996).³Stone et al. (1995).⁴Buitkamp et al. (1996).

BB702, and *BOBT24* showed 3 to 12 alleles. Both applied tests revealed no significant differences in microsatellite allele frequencies between the 2 groups of Holstein cows testing positive or negative for paratuberculosis. Microsatellite *BMC9006* showed only 3 alleles, 2 of which displayed very low frequencies in the present data set. This finding is in accordance with that of Paixao et al. (2006), who investigated 81 *Bos taurus* (Holstein) cattle and 167 animals from *Bos indicus* breeds with regard to brucellosis resistance and detected 100% of Holsteins homozygous for one allele, (GT)13. A survey of *B. indicus* (Haryana) and *B. indicus* × *B. taurus* crossbred cattle for the (GT)13 repeat in the 3' untranslated region of *SLC11A1* also revealed only homozygotes (Kumar et al., 2005). Our results are based on ELISA tests (specificities: IDEXX = 94%; Svanovir = 76%), which could lead to inappropriate classification of MAP-negative (control) animals. The main problem for genetic studies regarding paratuberculosis

is the definition of infection and disease status, resulting in difficult acquisition of material (Reddacliff et al., 2005). Paratuberculosis infection can be divided into 3 stages, noninfected, infected with predominating cell-mediated immune response (and therefore low humoral antibody response), and infected with predominating humoral antibody response and elevated antibody titers (Nielsen et al., 2002a). Some infected cows never progress to a clinical state accompanied by typical symptoms and no test can indicate whether an infected animal will later develop clinical paratuberculosis. The sensitivity of diagnostic tests for paratuberculosis is low and depends on the stage of disease (Nielsen et al., 2002b). The existence of breed-specific differences in susceptibility to infectious diseases in cattle has not been adequately addressed (Barthel et al., 2000). Additional diagnostic measures such as fecal culture and expanded sampling may give further insight into the contribution

Table 2. Raw means and standard deviations of all animals, the *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-positive group, and the control group for milk production traits per lactation¹

Milk production trait	All animals (n = 4,590)		MAP-positive group (n = 580)		Control group (n = 574)	
	Mean	SD	Mean	SD	Mean	SD
Yield (kg)	8,338	1,784	8,281	1,599	8,455	1,791
Fat (kg)	335.3	69.2	325.2	61.7	335.1	69.0
Protein (kg)	281.4	55.2	278.5	49.9	283.8	56.1

¹Only lactations with more than 250 DIM were included.

Table 3. Number of animals genotyped (n) and allele frequencies (%) of microsatellites *BB704*, *BMC9006*, *BMS1617*, *BOBT24*, *BB719*, *BB702*, *BB717*, and *BB705* of *Mycobacterium avium* ssp. *paratuberculosis* (MAP)-positive animals (positive), control group (negative), and results of χ^2 test and Fisher's exact test

Group	n	Allele frequencies (%)											
		<i>BB704</i> ^{NS}											
		118 ¹	120 ¹	146	148	150 ¹	154	158					
Positive	578	1.6	0.1	0.3	45.2	0.4	9.8	42.6					
Negative	571	2.1	0.0	0.7	41.9	0.1	11.4	43.8					
		<i>BMC9006</i> ^{NS}											
		229 ¹	231	235 ¹									
Positive	418	2.3	97.6	0.1									
Negative	572	1.7	98.2	0.1									
		<i>BMS1617</i> ^{NS}											
		148	152 ¹	154 ¹	156	158	160	162	164 ¹	166 ¹			
Positive	579	41.9	0.7	3.2	28.3	0.3	16.2	4.7	3.5	1.2			
Negative	573	42.7	0.7	1.7	28.8	0.1	14.5	5.8	3.8	1.9			
		<i>BOBT24</i> ^{NS}											
		151 ¹	153	155 ¹	157 ¹	159 ¹	161						
Positive	579	2.7	64.7	2.3	5.8	1.5	23.0						
Negative	573	1.7	64.6	2.4	4.1	1.5	25.7						
		<i>BB719</i> ^{NS}											
		258 ¹	264	266 ¹	268 ¹	276 ¹	278 ¹	280	284 ¹	286	288 ¹	290 ¹	292
Positive	575	0.4	23.4	3.6	0.1	1.1	3.8	12.2	1.5	39.7	0.5	0.4	13.3
Negative	568	0.5	20.8	3.9	0.0	0.5	4.3	12.0	1.3	44.0	0.2	0.5	12.0
		<i>BB702</i> ^{NS}											
		183 ¹	187	189	193	195 ¹	197	203 ¹					
Positive	583	0.3	5.5	7.0	74.8	0.4	7.6	4.4					
Negative	574	0.5	6.0	6.7	74.7	0.3	7.9	3.9					
		<i>BB717</i> ^{NS}											
		158 ¹	164	166 ¹	168	170 ¹	174 ¹						
Positive	571	0.4	86.1	3.7	9.0	0.5	0.4						
Negative	559	0.5	85.4	3.8	9.9	0.2	0.2						
		<i>BB705</i> ^{NS}											
		118	122	124									
Positive	582	90.7	7.7	1.6									
Negative	574	89.6	8.9	1.5									

¹Rare alleles with frequencies below 5% in the total sample were combined for statistical evaluation.

^{NS} = not significant.

of the investigated microsatellites to paratuberculosis susceptibility in German Holstein cattle.

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