NRAMP1 GENE EFFECT ON BOVINE TUBERCULOSIS BY MICROSATELLITE MARKERS ANALYSIS

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INTRODUCTION

One of the tasks of genetic improvement is to select animals resistant to infectious diseases, especially those difficult and expensive to eradicate, in order to obtain healthy animals in which endogenous potentiality is optimised and therapeutic events reduced. Out of these kind of infections are those caused by intracellular bacteria like *Brucella, Mycobacteria* and *Salmonella* which hold extreme importance both for the incidence and for the effects on human and animal health. In Italy, despite eradication programs, bovine Tuberculosis is still a sanitary and economic problem and the possibility to find a gene for susceptibility or resistance to the disease to be used for selection is quite promising.

Recently *NRAMP1* gene (Natural Resistance Associate Protein 1) has been identified as candidate gene for natural resistance/susceptibility to intracellular bacteria in mice (Vidal S. *et al.*, 1993) and men (Cellier M. *et al*., 1994). Structurally, *NRAMP1* gene has several exons mapping on chromosome 2 in men, chr.1 in mice, chr.7 in chickens, chr.15 in pigs and chr.2 in cattle. It codes for a membrane protein expressed only on monocyte-macrophages, structurally homologous to the ATP transport membrane proteins. After bacterial phagocytosis, *NRAMP1* concentrates on macrophage phagosome membrane where affects pathogen replication by bivalent cations regulation (Skamene *et al*., 1998 ; Kovarova *et al.,* 1998). Several gene polymorphisms have been described and their effect on gene function has been suggested (White *et al*., 1994 ; Blackwell *et al.,* 1995 ; Liu *et al*., 1995 ; Lewis *et al*., 1996). Strong evidences of direct effect of the gene on resistance/susceptibility to Tuberculosis in mice and men have been recorded (Skamene, 1994) and some allelic variants of the *locus* have been shown associated to Tuberculosis and Leprosis in man (Govoni *et al*., 1998). On a population studied by microsatellites, four polymorphic patterns in *linkage* with *NRAMP1* gene showed significant association with Tuberculosis susceptibility (Bellamy *et al*., 1998). Hypothesis of *linkage disequilibrium* between variation in *NRAMP1* gene and another nearby susceptibility gene has been considered unlikely by the authors. In cattle polymorphism has been shown only by SSCP (Single Strand Conformation Polymorphism) in studies on association with Salmonellosis and Brucellosis (Adams *et al*., 1998).

This study is aimed to identify associations between *NRAMP1* polymorphisms and the evolution of bovine Tuberculosis (TB) in a case-control study in TB infected farms on a wide sample of cattle by mean of microsatellite markers.

MATERIAL AND METHODS

Animals. A sample of 135 Holstein Friesian cattle has been collected in five Northern Italian farms declared infected or under clearance / reclamation by intradermal test (IDT).

Affected and control animals have been identified first by tuberculin skin test and later by γ-interferon (γ-IFN) titration performed by Italian Veterinary Service and by the Laboratory of the "Istituto Zooprofilattico della Lombardia ed Emila" respectively. The γ-interferon (γ-IFN) test has been used to determine infection status in order to overtake the low reliability of intradermal test (IDT) (Wood *et al*., 1990). In the sample the number of affected animals was comparable to the number of unaffected ones.

γ-interferon test. Peripheral blood mononuclear cells were grown *in vitro* for 24 h in the presence of reference bovine, avian PPD (Protein Purified Derivative) and PBS solutions. The γ-IFN release in the supernatant was assessed by a commercial kit and results have been expressed as Optical Density (OD) (Bovigam Test Kit, CSL) (Archetti *et al*., 1996).

Microsatellites. Two bovine and one ovine microsatellites mapping in close *linkage* with *NRAMP1* gene have been chosen : *BM64444* $(GT)_{16}$, *AR028* $(GT)_{19}$ (Bishop *et al*., 1994 ; Avraham *et al*., 1993) and *OVINRA1* $(GT)_{23}$ (Pitel *et al*., 1996) respectively. A fourth bovine microsatellite, *HORIN* (GT_{12} and GT_{13}) (Horin *et al*., 1999) in the 3'UTR region of *NRAMP1* gene has been considered. Microsatellites have been amplified by fluorescent primers according to author's instructions using an MJ PCR machine and electrophoresed on ABI Prism 377 (Perkin Elmer).

Statistical analysis. The effects of individual alleles, genotypes and homozygous individuals versus heterozygous on γ-interferon production have been analysed by multiple regression analysis using SAS package (Zanotti *et al*., 1995). The γ-interferon production level has been included in the model as dependent variable both as the ratio between the OD value of bovine-PPD-stimulated wells and OD of PBS control (bPPD/PBS), both as ratio between the OD value of bovine-PPD-stimulated wells and OD of avian-PPD-stimulated wells (bPPD/aPPD), both as the difference between bovine-PPD-stimulated wells and avian-PPD-stimulated wells (DIFF). Age and farm were included in the model as covariates. Allele and genotype classes with less than 0.05 frequency have been pooled in one class.

Table 1. Allelic and genotypic frequencies by locus

BM6444				AR028				HORIN	
alleles	**freq.**	**genotypes**	**freq**	**alleles**	**freq**	**genotypes**	**freq**	**alleles**	**freq**
150	0.437	150/150	0.200	260	0.011	260/262	0.015	211	0.155
152	0.504	150/152	0.422	262	0.504	260/264	0.007	213	0.845
154	0.059	150/154	0.052	264	0.341	262/262	0.244		
		152/152	0.259	266	0.041	262/264	0.356		
		152/154	0.067	268	0.033	262/266	0.045		
				270	0.066	262/268	0.045		
				280	0.004	262/270	0.052		
						264/264	0.104		
						264/266	0.030		
						264/268	0.022		
						264/270	0.059		
						264/280	0.007		
						266/270	0.007		
						270/270	0.007		

RESULTS AND DISCUSSION

No polymorphism has been identified for *OVINRA1*, while we could find seven alleles for *AR028*, two alleles for *HORIN* and three for *BM6444* over the 135 samples. Allelic and genotypic frequencies are given on table 1.

No effect was observed for any alleles at any locus, while the genotype 152/154 of *BM644* microsatellite affected significantly and positively bPPD/PBS and 262/270 of *AR028* microsatellite affected bPPD/aPPD and DIFF negatively (figure 1). Farm and age were not statistically significant and no significant effect was observed for heterozygous genotypes versus homozygous.

Since no allele of the three informative microsatellite loci showed any significant effect on γ-interferon production, we can assume that no one can be considered as a major gene affecting TB resistance/susceptibility in cattle. On the contrary single genotypes affected γ-interferon production. Particularly the genotype 152/154 of *BM644* seems to affect susceptibility to TB and 262/270 of *AR028* resistance. Only in the latter case the effect was more than one standard deviation.

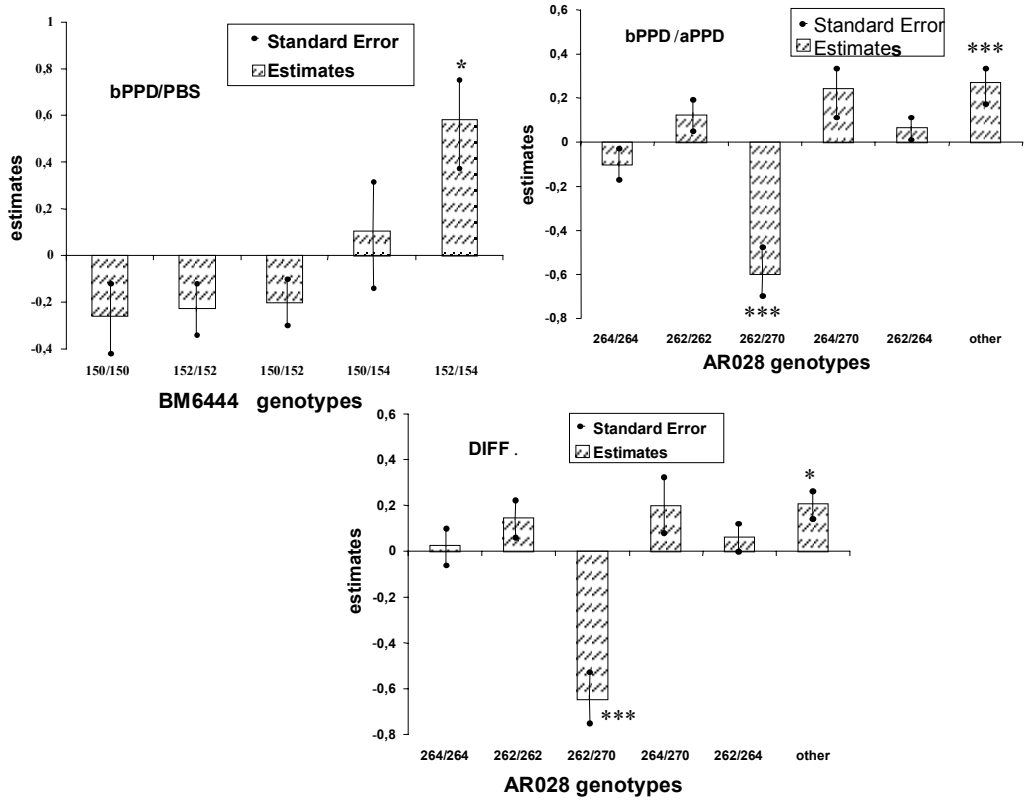

Figure 1. Effect of genotypes on bPPD/PBS, bPPD/aPPD and DIFF, standard error and probability (* = P < 0.001 ; * = P < 0.05)**

CONCLUSIONS

The results reported here, together with the data available in man and mice, support the hypothesis of an involvement of *NRAMP1* gene in the modulation of cattle immune response to *M. bovis* which needs to be confirmed, however, by studies on the sequence of the gene and on its function.

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