On the Search for Markers of Tick Resistance in Bovines

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Abstract: Genetic differences in susceptibility to ticks (*Rhipicephalus (Boophilus) microplus*) are considerable in bovines. Here, mapping, association and gene expression approaches were employed to further advance our understanding of the molecular basis of tick resistance. A *B. taurus x B. indicus* F_2 population was developed by Embrapa and 382 individuals were measured for parasitic load. Scanning of all chromosomes is in progress. Quantitative trait loci (QTL) for tick load were mapped to chromosomes 4, 5, 7, 10, 14, 18 and 23 out of the 20 chromosomes scanned and were dependent on the season in which the phenotype was scored. In the candidate gene approach, females from the genetic groups Nelore (NE - 184), Canchim x Nelore (CN - 153), Aberdeen Angus x Nelore (AN - 123) and Simmental x Nelore (SN - 120) were evaluated under natural infestation. Microsatellite markers close to the genes for interleukin 2 (*IL2*), interleukin 4 (*IL4*) and interferon gamma (*IFNG*) were analysed. Tick counts were associated with the marker for interleukin 4 ($P<0.05$) in three genetic groups. Differences in cytokine mRNA levels of naïve versus infested Nelore calves as well as between resistant versus susceptible cows from NE, CN and AN genetic groups were also investigated. Comparison of cytokines from infested and naïve animals showed downregulation of *IL2*. When resistant cows were compared to susceptible animals, *IL8* was downregulated. These results reinforce the multiloci nature of tick resistance and the need to consider QTL and environment interactions.

INTRODUCTION

The bovine tick *Rhipicephalus* (*Boophilus*) *microplus* and tick-borne diseases result in significant economic losses in cattle production in tropical and subtropical areas. The scenario becomes even worse with the selection of chemical resistant parasites and the increased public demand for residue free animal products. Host resistance to ticks is a complex trait influenced by a great number of environmental and physiological factors, such as temperature, humidity, gender and age [1]. Regardless, there is a genetic component of tick resistance with estimates of heritability varying from very low to high, according to evaluation method (artificial or natural challenge), study population and statistical method. Several studies have reported an association of genetic markers with tick count in different bovine breeds [1-3], suggesting that a portion of genetic control of tick resistance could be determined and used in the selection of resistant cattle.

MATERIALS AND METHODS

For the interval analysis in the F_2 generation, a *Bos taurus* (Holstein) x *Bos indicus* (Gyr) population of 382 F_2 animals was developed from 1999 to 2005. The F_2 generation was evaluated for tick resistance by experimental challenge in two seasons, as previously described [4]. Microsatellite markers were selected to cover 20 chromosomes at 20 cM intervals and linkage maps were assembled. Significance of the fixed effects and covariables for each trait was evaluated by least square analysis of tick count transformed to $\log_{10}$ (n + 1), where n is the number of ticks. Quantitative trait loci (QTL) analysis was performed using regression interval mapping for F_2 families [5]. The model included the additive and dominance effects of the QTL and all fixed effects (sex, year in which the animal was evaluated, coat color and hair type) that were significant in the analysis of variance (ANOVA). Age of the animal (days) at evaluation was included as a covariable.

For the candidate gene experiment, females with different physiological status (calves, pregnant and open heifers, primiparous and pluriparous cows with and without a calf) were selected from the following genetic groups (GG): Nelore (NE – n=184), Canchim x Nelore (CN – n=153), Aberdeen Angus x Nelore (AN – n=123) and Simmental x Nelore (SN – n=120). They were evaluated under natural infestation conditions in seven to ten tick counts, from July 2003 to December 2004. Animals were raised and evaluated at the Southeastern Embrapa Cattle Station, located in São Carlos, São Paulo, Brazil. During the entire evaluation period, animals were maintained on pastures, with no parasite control. Ticks 4.5 mm or larger were counted on one side of the animals, and the data transformed to $\log_{10}$ (n + 1). The transformed tick count, adjusted for genetic group (GG), animal within GG, year-season (YS) and physiological status, was obtained for each animal that had at least seven observations. DNA samples from the evaluated animals were part of a DNA repository held at the Southeastern Embrapa Cattle Station. Microsatellite markers were chosen based on their proximity to candidate genes from the bovine immune system, *BL4* (interferon gamma), *IL4* (interleukin 4) and *BMS941* (interleukin 2) and genotyped using an ABI3100. Adjusted tick counts estimated for each animal were used in least square analysis considering the fixed effects of sire, number of observations (7 to 10) and marker genotype. Low frequency genotypes (<4 animals) were excluded from the analysis. Since GG had different allelic frequencies, association analysis was done within GGs.

The experiment for measuring cytokine mRNA levels of naïve versus infested Nelore was held at the Southeastern Embrapa Cattle Station in Brazil (22°01'S and 47°53'W). Ten calves from ten different mothers were born in isolated pens, and kept free of ticks along with their mothers under intensive chemical control. When calves were four months old, they were assigned to two groups, reference and challenge. Paternal half-sibs were assigned each to a different group. Animals from the challenge group (five calves) were left without chemical tick control for 30 days before they were artificially infested with aliquots of *R. microplus* larvae, spread on the back of each animal. On the ninth day after tick infestation, samples from skin and inguinal lymph node were surgically collected using local anaesthesia. Total RNA was isolated using TRIzol® reagent (Invitrogen) and synthesis of complementary DNA (cDNA) was performed from 5.0 µg total RNA using oligo(dT) primer and Superscript II (Invitrogen). RT-PCR primers were designed [6] and real-time PCR was performed using SYBR Green I in a LightCycler™ (Roche Diagnostics, Mannheim, Germany), with *RPL19* (ribosomal protein L-19) as housekeeping gene. To calculate the relative expression ratio between reference and challenge groups, the Relative Expression Software Tool (REST)© [7] was used.

To determine cytokine mRNA levels of resistant versus susceptible bovines, ten cows from each genetic group (5 resistant and 5 suceptible) were selected from the candidate gene experiment to represent the extreme phenotypes of each genetic group. Animals were kept under chemical tick control for three months and left without chemical tick control for 30 days before they were submitted to artificial infestation. Tick challenge, tissue collection, RNA extraction and RT-PCR were as described for the naïve versus infested experiment.

RESULTS

In the F_2 experiment, the average tick count value was 40 ± 72.4 for the rainy season, with eight animals presenting more than 200 ticks, out of 302. The maximum observed value was 792 ticks in one animal, and 25 animals were free of ticks during this season. In the dry season, an average of 33 ± 43.3 ticks per animal was observed. The maximum observed value in one animal was 412, four animals had more than 200 ticks, and ten were completely free of ticks, out of 338 evaluated. Year of evaluation and hair type were significant for tick counts obtained during the rainy season ($P<0.01$). For the dry season, tick count was affected by coat colour and hair type ($P<0.01$). Interval analysis of F_2 has covered 20 chromosomes so far. QTL have been detected on chromosomes 4, 5, 7, 10, 11, 14, 18 and 23 (Table 1). Dominance deviations were significant for QTL on chromosomes 4, 5, 7, 14 and 18. Different QTL were found for phenotypes scored during the rainy or dry season. Altogether, QTL explained 13.07% of the total phenotypic variation for tick count during the rainy season and 11.28% of the total phenotypic variation for tick count during the dry season.

Table 1: Summary of QTL for tick resistance identified in the Holstein x Gyr F_2 population.

Chromosome	Season[1]	Significance	Position (cM)	d^2	%$\delta^2 P^3$
4	Rainy	5%	98	yes	2.40
5	Rainy	5%	132	yes	1.70
7	Dry	10%	73	yes	1.90
10	Dry	1%	18	no	6.20
11	Rainy	5%	32	no	3.40
14	Dry	5%	25	yes	3.20
18	Rainy	1%	60	yes	1.97
23	Rainy*	5%	50	no	3.60

[1] Season in which ticks were counted.
[2] Dominance deviation.
3 Proportion of the phenotypic variance explained by the QTL.
* This QTL was not tested for dry season data.

In the candidate gene experiment, the difference in resistance among genetic groups was dependent on the year and month of evaluation, but in all cases NE cows were less infested than all the other genetic groups. Of the molecular markers investigated, only the microsatellite linked to the IL4 microsatellite marker was associated ($P<0.05$) with adjusted tick count in three of the genetic groups in the study (NE, CN and AN). Genotype means are presented in Table 2. Since most of the genotypic combinations were not represented, it was not possible to assess allele substitution effects. There was no clear pattern of genotypes associated with higher or lower tick loads within or among genetic groups.

Table 2: Means and standard deviations for microsatellite IL-4 genotypes within genetic groups.

	Nelore		½ Canchim ½ Nelore		½ Angus ½ Nelore	
Genotype	Mean[1]	± SE	Mean[1]	± SE	Mean[1]	± SE
81089	-	-	0.527[b]	± 0.266	-	-
85093	-	-	1.219[a]	± 0.142	-	-
89089	0.699[a]	± 0.104	1.131[a,c]	± 0.209	-	-
89091	0.266[a,b]	± 0.160	1.236[a]	± 0.184	-	-
89093	0.478[a,b]	± 0.112	1.201[c]	± 0.119	1.343[b]	± 0.096
89105	0.412[b]	± 0.090	0.586[a]	± 0.186	-	-
91091	0.472[a,b]	± 0.155	-	-	-	-
91093	0.383[a,b]	± 0.172	0.689[a,c]	± 0.241	1.772[a]	± 0.150
91097	-	-	1.033[a,c]	± 0.413	-	-
91101	-	-	1537[a]	± 0.296	-	-
91105	0.685[a]	± 0.095	-	-	-	-
93093	0.414[a,b]	± 0.116	-	-	1.621[a,b]	± 0.112
93097	-	-	0.977[a]	± 0.423	-	-
93105	0.513[a,b]	± 0.101	1.286[a,c]	± 0.160	1.500[a,b]	± 0.102
105105	0.422[a,b]	± 0.100	1.898[a,c]	± 0.373	-	-

[a;b;c] Values with different letters within columns are significantly different ($P<0.01$).

Analysis of cytokine mRNA abundance in challenged compared to naïve Nelore calves (Fig.1) showed downregulation of *IL2* ($P<0.05$) mRNA in challenged animals, while no effect of tick challenge was observed for *IL4, IL8, IL12p35, TNF-α* and *MCP-1*. When extremes of tick resistance were compared across all genetic groups (Fig.1), downregulation ($P<0.05$) of *IL8* was observed in resistant cows. When the same comparison was performed within the different genetic groups, downregulation was observed only in the AN group ($P<0.01$) (data not shown).

DISCUSSION

The number of QTL found in the F_2 resource population suggests a multiloci nature for host resistance. Although tick resistance was evaluated by artificial challenge in order to minimize environmental effects, all the QTLs in question were dependent on the season in which the phenotype was scored. If tick counts were only performed during the rainy season, two QTL would not have been detected. This dependency could be related to seasonal variations in coat length and thickness, nutritional status, circadian rhythm or heat stress. The genotype-environment interaction could imply that animals selected from one environment would perform poorly in another. Moreover, the sampling problem associated with seasonal variation suggests the necessity for testing across many years.

Results for IL4 microsatellite association to ticks did not allow for the identification of a specific allele for resistance. This gene is located in the same region on chromosome 7 as a suggestive dominant QTL that was detected, between microsatellite markers IL4 and BM6117, in the F_2 experiment. Investigation of SNPs in the *IL4* gene could be helpful for confirming and understanding this association.
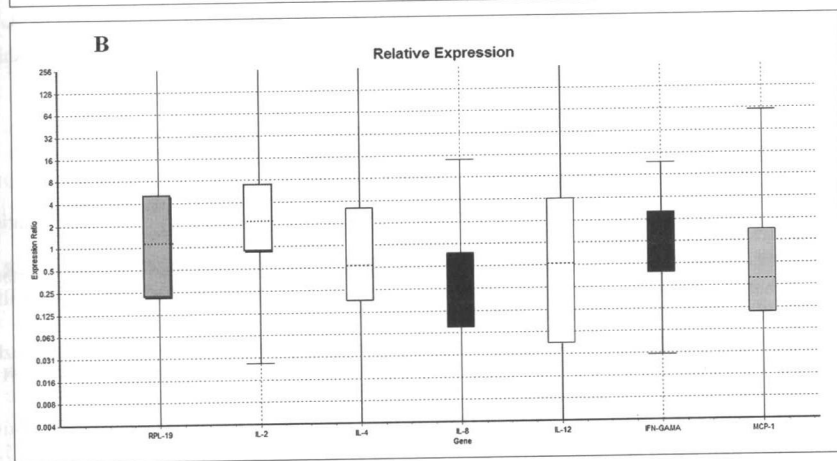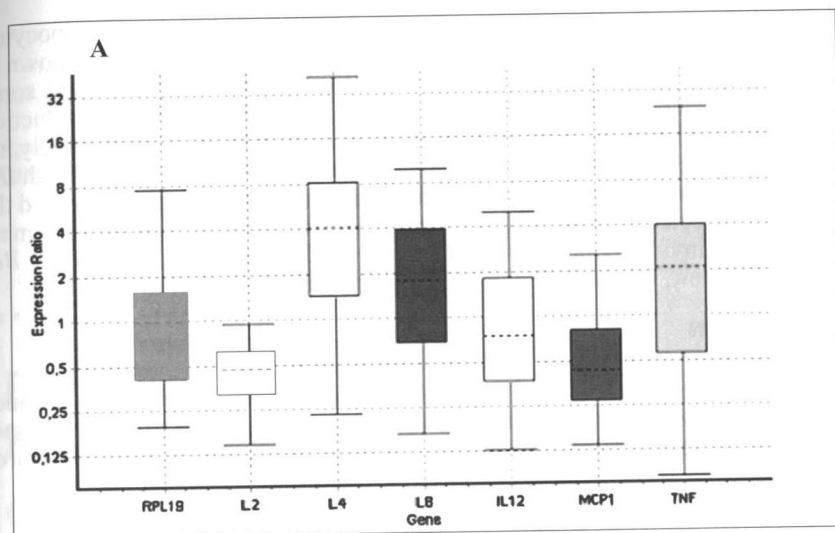
Fig. 1: Cytokine mRNA profile in lymph nodes. **A.** Relative expression of infested against naïve Nelore Calves. **B.** Relative expression of resistant compared to susceptible cows. Boxes represent the interquartile range, or the middle 50% of observations expressed as the n-fold differences between groups (A=Infested X Naïve, B= Resistant X Susceptible) in relation to the housekeeping gene. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations (some are out of range not displayed).

The cytokine IL2 is a growth factor that guides the proliferation and differentiation of T cells, and is self-produced by activated T cells. In mice infested with *Rhipicephalus sanguineus* ticks, *IL2* and *IFNG* were found to be largely downregulated, whereas *IL4* and *IL10* were upregulated [8]. We have shown that *IL2* is downregulated after a first challenge in Nelore calves, a resistant breed, with no difference between resistant and susceptible cows, which suggests that this cytokine may not play a main role in host resistance. IL8 is a chemokine that act mainly as a chemo-attractant for leukocytes, recruiting monocytes, neutrophils and other effector blood cells to

infection sites. It is produced by monocytes, macrophages, fibroblasts and keratinocytes. Salivary gland extracts (SGE) from several ixodid tick species have been shown to reduce IL8 levels through the presence of IL-8 binding proteins in SGE of some species [9]. According to our results, in *R. microplus* infested cows, the reduction would occur at the mRNA level and in a breed specific pattern. Unfortunately, no relationship between expression patterns and marker data could be drawn. Both *IL2* and *IL8* are on chromosome 6, which was not scanned in the F_2 experiment, and the microsatellite selected to mark *IL2* segregation in the candidate gene experiment turned out to be invalid, since it was selected based on the first build of the *Bos taurus* genome view, in which *IL2* was mapped to chromosome 17.

CONCLUSION

The marker data presented here suggests multiloci inheritance for tick resistance in bovines, with the predominance of non-additive effects and a strong genotype-environment interaction. Differences in gene expression of resistant cows compared to susceptible cows were breed-specific.

ACKNOWLEDGEMENTS

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