Short communication

Genetic analyses of polymorphisms on ovine chromosomes 5 and 20 and their effect on resistance to internal parasites

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Abstract

A trial was conducted to evaluate the association between polymorphisms in, or close to, candidate genes and faecal egg counts (FEC) in sheep. Corriedale and Polwarth weaned ewe lambs from commercial flocks were submitted to three successive experimental challenges in naturally contaminated pastures in Southern Brazil. During the trial, \textit{Haemonchus contortus} was the predominant parasite species. Individuals were phenotyped according to their post-challenge FEC averages. Polymorphisms at interleukin genes (IL-3, IL-4 and IL-5), located at sheep chromosome 5 (OAR5) and the microsatellite markers BM1815, CSRD226, OarHH56, and OMHCI, located at sheep chromosome 20 (OAR20), were tested. A forced PCR-RFLP method to genotype ovine IL-5 was also developed and described. Associations between phenotypes and genotypes were tested by allele substitution analyses. There was no significant association between logFEC and the polymorphisms examined here, except for IL-4 in the Corriedale population. Results showed that FEC averages were significantly reduced when IL-4*\textsuperscript{B} allele, the most frequent allele, was replaced by either IL-4*\textsuperscript{A} or IL-4*\textsuperscript{C} alleles (\(P<0.05\)).

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1. Introduction

Internal parasite infections are one of the most limiting factors for sheep production worldwide. In the last decades, indiscriminate use of anthelmintic drugs led to parasite resistance, worsening the problem. Amongst the alternative methods found to control internal parasite infections, selection of resistant sheep based on faecal egg counts (FEC) is being regarded as a possible option to increase the frequency of more resistant individuals and to improve sheep production as a whole. Sheep breed differences concerning resistance to internal parasites have been reported in Barbados Blackbelly, Navajo, Florida Native, St Croix (Knight et al., 1973; Courtney et al., 1984; Gamble and Zajac, 1992), and in the Red Maasai (Preston and Allonby, 1978, 1979; Mugambi et al., 1997; Baker et al., 2003) breeds, when compared to wool and meat sheep breeds. These native populations have been bred in highly parasite-contaminated areas, therefore their better resistance to withstand parasite infections might have been developed as these flocks co-evolved with internal parasite infections.

Faecal egg count is based on a simple and low cost procedure. It is the current technique to determine the level of internal parasite infections in flocks. Therefore, several animal breeding programmes are using FEC as indirect selection criteria to select for resistant sheep to internal parasites. The estimated heritability values for FEC vary between 0.14 and 0.44 (Piper et al., 1987; Watson et al., 1986; Baker et al., 1991; McEwan et al., 1992; Morris et al., 1997). The main drawback is that FEC is only expressed in sheep under challenge conditions, which translates to high costs to phenotypically assess different levels of resistance in all individuals from the mob. Adding to this,
post-weaning challenges can negatively affect lifetime production, especially on more susceptible animals.

Finding molecular markers associated to resistance/susceptibility to internal parasite infections would allow farmers to select desirable genotypes at an earlier age, thus reducing production costs and helping researchers to identify immunological mechanisms responsible for host resistance to internal parasite infections. The association between molecular markers and resistant phenotypes has been studied since the 80’s. Markers located close to the ovine major histocompatibility complex (OMHC) chromosomal region were the first to be studied because these genes are involved in immunological induction and regulation and have the advantage of being highly polymorphic.

Result studies are not unanimous, some have found no significant effect of MHC genes on the susceptibility of sheep to internal parasites (Cooper et al., 1989; Crawford et al., 1997), some others have reported significant associations between FEC reductions and (a) DRB1 alleles in Scottish Blackface lambs naturally infected with Teladorsagia circumcincta (Schwaiger et al., 1995), (b) MHC Class I and DY (Class II) alleles (Buitkamp et al., 1996) in the same previous flock, (c) OLADRB alleles in unmanaged Soay sheep infected with T. circumcincta (Paterson et al., 1998), (d) OarCP73 and BM1815 alleles (markers located close to the OLADRB gene) in Rhönschaf sheep naturally challenged with Haemonchus contortus (Janßen et al., 2004), and (e) Ovar-DRB1 alleles in Suffolk, but not in Texel sheep, infected with Trichostrongylus (Sayers et al., 2005a).

As host responses to internal parasite infections depend on complex immune system mechanisms, it is likely that genes other than those from the MHC complex might also be involved in resistance to internal parasites. In fact, polymorphisms at markers closely located to the interferon gamma gene (IFNγ) region have been associated to sheep response to internal parasite infections (Crawford and McEwan, 1998; Paterson et al., 2001; Coltman et al., 2001). This region was also confirmed by a genome-wide scanning study on half-sib sheep originated from divergent selection for high and low resistance to Trichostrongylus colubriformis infection (Beh et al., 2002). More recently, a microsatellite and a SNP markers located at the IFNγ intron 1 have been reported to be associated to host resistance to Trichostrongyloide infection in Texel, but not in Suffolk sheep (Sayers et al., 2005b). Interferon gamma is a Th1 cytokine protein that antagonises Th2 responses, which are important to build humoral responses against internal parasite infections.

Other chromosomal regions have been found to be associated to sheep resistance to internal parasite infections in the Ovis aries (OAR) chromosome 1 (Beh et al., 2002; Diez-Tascón et al., 2002), OAR6, OAR11, and OAR12 (Beh et al., 2002). Significant associations in Corriedale and Polwarth commercial flocks were found between FEC reductions after natural mixed parasite challenges (H. contortus being the predominant species) and CSRD2138 alleles (Benavides et al., 2002). The latter marker is located at the OARS chromosome, close to the interleukin 3, 4, and 5 genes (Maddock et al., 2001) which are responsible for IgA and IgE isotropic expression changes, clonal development and maturation of these immunoglobulins and eosinophil. This is a follow-up study to confirm the association between known polymorphisms at either intronic or exonic regions at IL-3, IL-4 and IL-5 and FEC in Corriedale and Polwarth commercial flocks. This work will also investigate the association between four OAR20 microsatellite markers and FEC in these flocks.

### 2. Material and methods

#### 2.1. Animals

Corriedale (n=48) and Polwarth (n=82) weaned ewe lambs, of approximately seven months old, from the Brazilian Agricultural Research Corporation (Empresa Brasileira de Pesquisa Agropecuária—Embrapa) South Cattle & Sheep Research Centre (54° 23’W and 30° 47’S) were used in the trial. These animals have not been previously selected for reduced FEC although efforts to select for wool production were made in the past.
Table 2

Molecular marker information: polymorphism, ovine chromosome location (OAR) and allele sizes.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Polymorphism</th>
<th>OAR</th>
<th>Allele size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM1815</td>
<td>Microsatellite</td>
<td>20</td>
<td>136–166</td>
</tr>
<tr>
<td>CSRD226</td>
<td>(CA)nTATAAA(CA)n repeat</td>
<td>20</td>
<td>149–195</td>
</tr>
<tr>
<td>IL-3</td>
<td>(CT)n repeat in intron 1</td>
<td>5</td>
<td>255 and 251</td>
</tr>
<tr>
<td>IL-4</td>
<td>C→T mutation in intron 1</td>
<td>5</td>
<td>SSCP</td>
</tr>
<tr>
<td>IL-5</td>
<td>G→C mutation in exon 4 causing silent mutation</td>
<td>5</td>
<td>138 and (108 + 30)</td>
</tr>
<tr>
<td>OarHH56</td>
<td>(AC)n repeat in intronic region</td>
<td>20</td>
<td>149–159</td>
</tr>
<tr>
<td>OMHCI</td>
<td>(CA)n repeat in intronic region</td>
<td>20</td>
<td>182–210</td>
</tr>
</tbody>
</table>

2.2. FEC phenotyping

Animals from these two breeds were raised as a single flock and challenged according to the methodology described in McEwan (1994). Briefly, animals kept on naturally contaminated paddocks were drenched to zero faecal egg counts. Faecal samples were collected on 5% of the individuals on a weekly basis. When FEC ranged between 800 and 1500, all animals were individually sampled and drenched again to resume a new challenge. This procedure was repeated two more times in early autumn (from March to May) to coincide with the epidemiological prevalence of *H. contortus* in the region. The extremely wet (total monthly rainfall ranged from 150.3 mm to 235.8 mm) and hot (monthly extreme temperature averages ranged from 19.8 °C to 27.4 °C) climatic conditions during these months resulted in better parasite survival on pasture. The number of nematode eggs per gram of faeces was determined by using a modified McMaster method (Whitlock, 1948). Faecal cultures were prepared as a pool of samples for each group. Infective larvae were identified according to Ueno and Gonçalves (1998) descriptions.

2.3. Genotyping

Lambs were blood sampled by venepucture for DNA extraction (Miller et al., 1988). DNA samples were amplified using the following molecular markers: BM1815, CSRD226, IL-3, IL-4, IL-5, OarHH56, and OMHCI. Molecular marker details were described in Tables 1 and 2. DNA amplification with microsatellite markers and with IL-3 and IL-4 was performed on a PerkinElmer 2400 thermocycler machine using PCR conditions described according to each reference.

PCR products of all markers, except IL-4 and IL-5, were analysed by electrophoresis in 1.2 mm-thick nondenaturing 10% polyacrylamide gels and fragment visualisation was done after dyeing gels on 0.5ng/ml ethidium bromide (Lahiri et al., 1997). Molecular weight markers were prepared by digesting pBR322 and dX174/Hinf I vectors with restriction endonucleases. Polyacrylamide gels were then photographed in a Kodak DC120 camera and fragment sizes analysed by using the Kodak Digital Science 1D software.

Interleukin 4 polymorphism was detected by using the SSCP technique where PCR products were denatured at 95 °C for 5 min and loaded with a formamide dye in a 1.2 mm-thick nondenaturing 6% polyacrylamide gels electrophoresed during 15h at 14 °C. Fragment visualisation was done after dying gels on silver nitrate.

2.4. Forced PCR-RFLP for sheep IL-5

A base substitution (G→C) was detected at nucleotide 336 bp (Maddox, personal communication) at the fourth exon of the Ovis aries interleukin 5 gene (GenBank accession number U17053) and SSCP has been used to identify this polymorphism. The primer sequences were: forward 5'-GAC AGT TTC CTA CGA TGC TTA TTG TC-3' (Maddox, personal communication) and reverse: 5'-CAG CTT TCC ATC GTC CAC TCT GTG TT GAT-3', with a G nucleotide designed to amplify a modified mutant type sequence so MboI restriction enzyme would be able to cut and differentiate it from the wild type (Fig. 1).

PCR conditions for IL-5 were: 80ng of genomic DNA amplified with 200 M of dNTPs, 2.5 l of 10× Taq DNA polymerase buffer (1.5 mM MgCl2), 0.4 mM of each primer and 2.5 U of Taq DNA polymerase to a final 25 l PCR reaction. The following PCR conditions were used: 94 °C (3 min) for one cycle, 95 °C (30 s), 50 °C (30 s), 72 °C (30 s) for 30 cycles, 72 °C (3 min) for one cycle. PCR fragments were digested with 10 µl of PCR products with 10U MboI and 1.5 l of MboI buffer in a 15 µl final reaction at 37 °C for 15 h. Digested fragments for this forced PCR were separated by 2.5% agarose electrophoresis and visualised by ethidium bromide staining.

**LOCUS** QAILV2

**DEFINITION** Ovis aries interleukin-5 (IL5) gene, exons 3, 4 and complete cds.

**ACCESSION** U17053

**VERSION** U17053.1  GI:897559

**KEYWORDS**

**SEGMENT** 2 of 2

**SOURCE** Ovis aries (sheep)

**ORIGIN**

1 ttatattactg tttttaaatc ttctatttta gcaccaacta tgcattggaag aagttctttca
61 gggaaacagc acatggaag atcaaacagtca acaaggatgt gcgtggaaaaa aataattcgc
121 aacattgtct ttaataaag aatatcataga cccattaanaa gtaaggtttaa aacatttattg
181 gcagaaacttat gtatattggt ctgtattgctgctgctgctgcttcttac gggaaattgac agtttcttcg
241 gacggtctatt gtctgtcttt tcacagagg aagttcttttgag gaaagagatg gattggaaga
301 caatttcgcg actacgcgcg agtttcttcg ggttctttata aacacaaggtg gagcagttgaa
361 caatttcgcg actacgcgcg agtttcttcg ggttctttata aacacaaggtg gagcagttgaa
cagctttgctggctctgcac gtcgctctcttc
361 agtggagact ttccttttttc actgctggtaa aagttctttgg ggaggaaggg gaggggaagttg cagtttaag
421 caaccaaggt gagggccacac caattgtggg

Fig. 1. Ovine interleukin 5 sequence (U17053). Different patterns correspond to: wild type nucleotide (grey), mutant nucleotide (box), forward primer (underlined), reverse primer (italics) and modified designed nucleotide in the reverse primer (capital).
Table 3
Descriptive statistics for the mean FEC and mean logFEC (mean ± S.D.) of the three successive challenges made in the two populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Mean logFEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corriedale</td>
<td>3.317 ± 1.736</td>
<td>567–10,366</td>
<td>3.31 ± 0.29</td>
</tr>
<tr>
<td>Polwarth</td>
<td>2.714 ± 1.005</td>
<td>733–7,266</td>
<td>3.27 ± 0.17</td>
</tr>
</tbody>
</table>

2.5. Statistical analyses

In order to confirm whether associations between alleles and FEC in the Corriedale population were not flock specific, molecular markers that showed significant effect in the Corriedale flock were tested on a second flock: a Polwarth population with two important characteristics: (a) it was field challenged with the Corriedales as a single mob, and (b) it had different allelic frequencies from the latter breed (Benavides et al., 2002), providing a reference flock for validation.

Allele frequencies between populations were compared by using a Chi-square test. Because small expected frequencies occurred in all comparisons, the P values were estimated by using the Monte Carlo solution of Roff and Bentzen (1989). Polymorphism information content (PIC) was calculated to determine the within flock genetic variability (Botstein et al., 1980).

Logarithmic transformation was used for the average FEC values (\[\log_{10}(\text{FEC} + 25)\]) prior to statistical analyses (average of FEC counts taken from the same animal at three different times). The constant value (25) was arbitrarily used in order to include zero FEC in the analyses. Allele substitution analyses were estimated by regression procedures under the PROC GLM with the solution option (SAS, 1990; Stear et al., 1989). All statistical analyses were independently run per molecular marker and breed. The statistical model used was:

\[Y_{ij} = \mu + g_i + m_k + e_{ij}\]

where \(Y_{ij}\) is the individual mean logFEC; \(\mu\) the population mean; \(m_k\), \(n_k, o_k\), are the scores for the proportion of each allele on the genotype; \(g_1-g_n\), the corresponding partial regression coefficients (gene substitution effects as deviations from the most frequent allele for each marker), and \(e_{ij}\), the residual error. Sire was not fitted as random effect due to the lack of pedigree information. Twinning percentage in both mobs was <0.77%.

3. Results

Faecal egg count averages for each population were presented in Table 3. There was no significant difference in logFEC mean (\(P>0.05\)) between populations. The predominant parasite genera was Haemonchus (65%), followed by Strongyloides (28%), Teladorsagia (4%) and Cooperia (3%).

No significant associations between logFEC and the polymorphisms analysed were observed. The only exception was for the IL-4 polymorphism in the Corriedale population. In this breed, the PIC value for this marker was 0.51 and the frequency of homozygous IL-4 ‘B’ animals was 25.5%. These results are striking when compared to a PIC value of 0.26 with 55.6% of homozygous IL-4 ‘B’ animals in the Polwarth population. Furthermore, Corriedale sheep showed five different IL-4 genotypes (AA, AB, AC, BB, and BC), compared to only three IL-4 genotypes for Polwarth sheep (AB, BB, and BC), which explains the high frequency of B alleles in the latter flock.

Allele substitution analyses showed that replacing IL-4 ‘B’ allele, the most frequent allele, by either IL-4 ‘A’ or IL-4 ‘C’ would reduce logFEC values (Fig. 2). Effects for this marker were not significant (\(P>0.05\)) in the Polwarth population though.

3.1. Forced PCR-RFLP for sheep IL-5

The developed technique allowed easy genotyping in agarose gels, after MboI digestion. Wild type sheep show 108 and 30 bp fragments and mutant individuals a 138 bp fragment (Fig. 3). Segregation was observed in three-generation families containing 18 individuals belonging to the International Mapping Flock (IMF). Inheritance of the two alleles was shown in Fig. 4.

4. Discussion

The high FEC values observed in this study are illustrative of the high gastrointestinal nematode infection levels shown in Southern Brazilian commercial flocks. These high egg counts also agree with the high egg output from the main parasite species H. contortus. Selection of resistant hosts to gastrointestinal nematodes can be seen as an alternative for internal parasite control. Among the molecular markers studied for sheep internal parasite resistance, regions from the sheep chromosome 20 (OAR20) have been the most extensively examined (Outteridge et al., 1985, 1986; Cooper et al., 1989; Schweiger et al., 1995; Buitkamp et al., 1996; Stear et al., 1996; Crawford et al., 1997; Paterson et al., 1998; Janßen et al., 2004; and Sayers et al., 2005).

This chromosome is the location of the major histocompatibility complex genes that play important role in host immunological response against internal parasites, and
some markers within this chromosomal region have been reported to be associated to sheep resistance to endoparasites (Outteridge et al., 1985; Douch and Outteridge, 1989; Schwaiger et al., 1995; Buitkamp et al., 1996; Patterson et al., 1998).

One of the most frequently studied markers, the DRB1 locus (alias OLADR), has been associated to FEC (Schwaiger et al., 1995; Stear et al., 1996; Paterson et al., 1998), and Sayers et al. (2005) for the Suffolk breed. However, other studies were unable to establish significant associations between DRB1 alleles and FEC (Crawford et al., 1997; Janßen et al., 2004), and Sayers et al. (2005) for the Texel breed. This marker is located at 25.5 cM from BM1815 and at 3.8 cM from OMHCI. Significant association results between BM1815 alleles and FEC were found in Rhônschaf sheep after *H. contortus* artificial challenge (Janßen et al., 2004) and between OMHCI alleles and FEC in a Scottish Blackface flock infected with *Teladorsagia circumcincta* (Buitkamp et al., 1996). On the other hand, the lack of association of BM1815 and OMHCI and FEC observed in this study, agrees with results found in the Soay sheep (Paterson et al., 1998). These markers had PIC values of 0.86 and 0.85 for the Corriedales, respectively and 0.85 and 0.89 for Polwarths, respectively. Likewise, our results showed no association between OARHH56 alleles and FEC, which is also in agreement with results found by Janßen et al. (2004).

Due to the complex immunological mechanisms involved in resistance to parasite infections, it would be highly likely that host resistance could be affected by several genes. Therefore, studies in other than MHC-related genes should be explored. Genome-wide scanning investigations confirmed that significant LOD scores have been shown between FEC and markers from the sheep chromosomes 1, 3, 6, 11 and 12 (Beh et al., 2002). Results from genome-wide scanning run on cattle exposed to *Ostertagia ostertagi* also found significant QTLs in bovine chromosome (BTA) 5 (syntenic to OAR3) and 6 (syntenic to OAR6) (Gasbarre et al., 2003). Furthermore, markers located close to the interferon gamma gene (IFNγ) region, at OAR3, have been previously reported to be associated to sheep response to internal parasite infections (Crawford and McEwan, 1998; Paterson et al., 2001; Colman et al., 2001).

The importance of Th2-type cytokines, especially high IL-4 have been reported by Pernthaner et al. (1997) who showed that IL-4 mRNA expression levels in the gastrointestinal lymphatic tissue were important for the immune responses in sheep genetically resistant to *T. colubriformis* after natural challenge. Mastocytosis, eosinophilia and high IgE levels, processes that characterise Th2 responses and are IL-3, IL-4 and IL-5 dependent, are the main mechanisms for mice defence against parasitic gastrointestinal nematodes (Finkelman et al., 1991; Finkelman and Urban, 1992; Finkelman et al., 1997).

Early results from Corriedale and Polwarth populations have indicated that the CDR2138 marker, an ovine chromosome 5 (OAR5) microsatellite marker located close to the IL-3, IL-4, and IL-5 genes, was significantly associated with FEC reductions. Animals bearing the CDR2138A allele showed 22% and 28% FEC reduction from the Polwarth and Corriedale flocks averages, respectively, after multiple helminthic species (mainly *H. contortus*) natural challenge (Benavides et al., 2002). CDR2138 marker is located at OAR5 (36.4 cM), i.e., in the same chromosomal region of the IL-4, IL-5 and IL-3 genes, therefore, the examination of polymorphisms in these interleukins was regarded as a way to test direct associations between FEC and these genes. The fact that a significant association was observed for a polymorphism in the intronic region of the IL-4 gene suggests that this specific marker might be used as a molecular marker in the Corriedale population. Polwarth results for the IL-4 intronic marker did not show the same trend as observed in the Corriedale flock. The high frequency of IL-4B6 genotype in this population might have negatively influenced the results.

Stear et al. (2007) discussed the factors that determine the power to detect statistical significance of genetic effects (in this case of MHC) on sheep resistance to nematodes. They state that, among others, the inclusion of sire and dam in the model as random effects helps decreasing the chance of spurious associations and increases statistical power. In our case, there was no pedigree information and the low number of phenotyped individuals indicates that our results must be interpreted with caution.

As the sheep genome is being mapped, the existence of other genes closely located to the IL-4 genomic region that might affect sheep resistance to internal parasites is still unknown. The results found here showed that IL-4A and IL-4C alleles reduced FEC counts and that this effect was limited to Corriedale sheep.

As a parallel result, a new technique consisting of a forced PCR-RFLP was attempted for ovine IL-5 (GenBank accession number U17053) by deliberately introducing a MboI site by the means of synthesising a reverse primer to complement a G base at position 339 rather than a T.
The objective of this methodology was to quickly genotype the sheep exon4/IL-5 marker in agaron gels, after MboI digestion (Fig. 3), replacing the current SSCP technique which is difficult to perform and requires specialised equipments. The advantages of this method are the speed of the analyses, from PCR to genotyping, estimated in 6 h when compared to 12 h of the current SSCP method, and the need of minimal optimisation of electrophoresis protocols compared to the lengthy optimisation needed to set up SSCP conditions (bis:acylamyde proportions and electrophoresis temperature and time). Interleukin 5 (IL-5) is an important cytokine responsible for eosinophils differentiation, recruitment and activation and it has been reported to be over-expressed in genetically resistant animals (Gill et al., 2000). Although silent mutations might not be considered as important as they cause no differences in protein expression, this polymorphism may be valuable as a molecular marker.

5. Conclusion

Allele substitution analyses showed that IL-4 A and IL-4 C alleles were associated to reductions in mean log FEC values in a small population size of Corriedales, but not in the Polwarth population.

Acknowledgements

We thank M. Noelle Cockett for kindly donating the primers used in the experiment, and Drs. Jill Maddox and Rachel Hawken for the IL-5 sequences and for testing the inheritance of the forced PCR-RFLP IL-5 in a three-generation family containing 18 individuals belonging to the International Mapping Flock (IMF). The authors also thank to CNPq, Embrapa, FAPERGS, FINEP, and IICA/PRODETAB for funding the project and scholarships.

References


