Detection of quantitative trait loci for internal parasite resistance in sheep. I. Linkage analysis in a Romney × Merino sheep backcross population

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SUMMARY

This study aimed to identify putative quantitative trait loci (QTL) that significantly affect internal parasite resistance in a backcross sheep population. A Romney × Merino backcross (to Merino) flock was challenged in 3 separate infections with Trichostrongylus colubriformis (primary and secondary) and Haemonchus contortus (tertiary). Haematological parameters were measured and faecal worm egg counts (FWEC) were established to estimate parasite burden. QTL mapping was conducted for FWEC and for the changes in haematocrit following H. contortus challenge and in eosinophil numbers following T. colubriformis challenge. Animals were genotyped for 55 microsatellite markers on selected chromosomes 2, 3, 6, 11, 13, 15, 21, and 22. Four putative quantitative trait loci were found; these being for eosinophil change in the primary infection (OAR 21), for FWEC in the first infection and eosinophil change in the secondary infection (OAR 3) and for FWEC in the secondary infection (OAR 22). No significant quantitative trait loci were detected for FWEC or haematocrit change during the Haemonchus contortus infection. The position of the putative quantitative trait loci for eosinophil change on OAR 3 is consistent with other reports of parasite resistance quantitative trait loci, implying some commonality between studies.

Key words: sheep, quantitative trait loci, eosinophils, erythrocytes.

INTRODUCTION

Genetic improvement programmes for internal parasite resistance in sheep were initiated in the early 1970s, after the first cases of helminth resistance to commercially available anthelmintics were reported (Coglazier et al. 1974). Since then, considerable research has shown that indirect infection traits, in particular faecal worm egg count (FWEC), have been used successfully to select sheep resistant to Haemonchus contortus and Trichostrongylus colubriformis (Windon et al. 1987; Woolaston et al. 1990; Karlsson et al. 1991). FWEC has been used most commonly, but other potential selection criteria include associated traits linked to immune responses such as change in blood eosinophils, antibody responses or predictive traits such as haemoglobin type or, for Haemonchus contortus pathology, the change in erythrocyte numbers measured as haematocrit or packed cell volume (Eady, 1995). Haematocrit change is a function of total blood loss due to worm burden and the sheep’s ability to replace erythrocytes through erythropoiesis (Albers et al. 1987).

Commercial implementation of genetic improvement programmes would benefit from alternatives to faecal worm egg counts (FWEC) and blood parameters, which are both time consuming and expensive to measure. There is a range of other problems with selection based on indirect measures of parasite burden such as FWEC (Hunt et al. 2008). For example, natural exposure can vary from year to year or, in a dry climate, can be completely absent, making collection of data in those years impossible. An artificial infection can standardize infections, but this is difficult to achieve in a commercial setting. Therefore, the application of gene marker tests or the detection of quantitative trait loci (QTL) that provide insight into the underlying pathways of internal parasite resistance would be highly beneficial for the sheep wool and meat industries.

There has been an international focus on QTL mapping for parasite resistance in sheep and a range of nematode species, infection protocols and breeds of sheep have been used (Dominik, 2005). Therefore, it is not surprising that few common findings have been discovered across the different studies. Most studies investigated either single parasite challenges or mixed species field infections. This study used a unique approach of multiple successive challenges with 2 nematode species that have differing pathological effects.

The aim of this study was to investigate the existence of putative QTL for internal parasite resistance in sheep. The mapping experiment was designed to allow comparison between nematode
species (*H. contortus* and *T. colubriformis*), different parasite resistance indicator traits and primary and secondary challenges in *T. colubriformis*.

**MATERIALS AND METHODS**

**The CSIRO Gene mapping flock**

In 1992, the CSIRO Gene mapping flock was established with the primary aim of mapping QTL for wool quality and production. For this purpose the Merino and Romney breeds that are divergent for wool characteristics were used. The 2 breeds also differ in their responses to parasites, and Romney parasite burdens are typically much less than those of Merinos kept under the same field conditions (Windon et al. 1993) and potentially genes that influence resistance to internal parasites segregate in this population.

Romney sires were crossed with Merino ewes to generate the F1 generation. Two F1 sires were used between 1992 and 1997 to produce groups of half-and full-siblings. No records on parasite challenges exist for these animals. In the years 2003 and 2004 one of the F1 sires, for which sufficient cryopreserved semen was available, was backcrossed to oestrus-synchronized Merino ewes by artificial insemination. In the two years, 399 progeny were born within 10 days. Phenotypes for parasite resistance indicator traits have been collected on these animals and they were used for analysis in this study.

**Phenotype data collection**

Gene mapping flock backcross animals were born in late spring of 2003 and 2004. After weaning at approximately 5 months of age, all animals were faecal sampled to assess parasite burden. Around 50% of lambs had low WFEC (whole flock median WFEC = 300 eggs/g) and all lambs were treated with anthelmintic drugs (Fenbendazole, Levamisole and Naphthalaphos at the manufacturer’s recommended dose). Lambs were then challenged with 3 orally administered parasite infections: a primary and secondary challenge with 20,000 McMaster susceptible strain *T. colubriformis* larvae (*Tc*) and a third challenge with 5000 Kirby1981 isolate *H. contortus* larvae (*He*). Each infection proceeded for 5 weeks before an anthelmintic treatment was used to clear the infection, followed by a 1-week break before the next challenge. At the end of the infection regime, animals were again treated with anthelmintics (Fenbendazole, Levamisole and Moxidectin according to the manufacturer’s recommended dose).

Faecal samples were taken from the rectum on days 21, 28 and 35 of each infection and FWEC assessed using the McMaster egg flotation technique for each sample. For each infection, the mean of the FWEC at the 3 sampling times was analysed (mean FWEC 1, 2 and 3). Peripheral blood was collected from the jugular vein into 10 ml vol. K$_2$EDTA Vacutainer® tubes. Five ml of blood was sampled for the primary infection on days 0, 14 and 30 (2003 born animals) and days 0, 12 and 26 (2004 born animals), for the secondary infection on days 0, 4, 13 and 28 (2003) and days 0, 4, 14 and 28 (2004) and for the tertiary infection days 0, 4, 15 and 28 (2003) and days 0, 4, 14 and 29 (2004). The blood was analysed using a Cell-Dyn 350R Autoanalyzer (Abbott) calibrated for ovine samples. The abundance of platelets and leukocytes and erythrocytes was recorded and the haematocrit (% blood volume comprised of erythrocytes) and eosinophil counts were analysed in this study.

The infection and sampling protocol was designed to resemble that used by Beh et al. (2002). Each parasite challenge was assessed using larvae cultured from pooled faeces and was found to consist predominantly of the intended parasite.

**Marker selection**

In total, 160 microsatellite markers were chosen for molecular characterization of the animals born in 2003. These markers were selected to be approximately 20 cM apart and spanned the whole genome. A whole genome scan on 200 animals born in 2003, analysing parasite resistance and wool phenotypes, detected significant QTL on chromosomes 2, 3, 6, 11, 13, 15, 21 and 22. Animals born in 2004 were selectively genotyped for 55 markers spread across these 8 chromosomes. Marker amplicons from the sire and grand-sire animals were assessed, and only those markers for which the sire was heterozygous were used for genotyping of their offspring. The genotypes of the Romney paternal grand-sire and in some cases the Merino paternal grand-dam were also assessed so that each paternally inherited allele in the offspring could be assigned as Romney or Merino. A third of the alleles could not be assigned to be paternal due to allelic similarities in the Romney and Merino breed. Details of all markers used in this study are available on the Australian Sheep Gene Mapping website (http://rubens.its.unimelb.edu.au/~jillm/jill.htm).

**Data preparation**

From the 399 animals of the CSIRO Gene mapping flock born in 2003 and 2004, 361 animals had phenotypic records and data on fixed effect and covariates. Mean faecal worm egg counts of the 3 sampling times (days 21, 28 and 35) for each of the 3 internal parasite infections (mean FWEC1, FWEC2 and FWEC3) were analysed. The change in erythrocyte numbers, expressed as the difference in haematocrit between day 0 and day 35 of the *H. contortus* infection (HetDiff), and the change in eosinophil
numbers as the difference between day 0 and day 28 of each *T. colubriformis* infection (EosDiff1 and EosDiff2) were also analysed. All blood cell parameters were correlated with mean FWEC1, mean FWEC2 and mean FWEC3 to prioritize the parasite resistance traits for analysis. Moderately strong negative correlations exist between erythrocyte parameters (red blood cell count, haemoglobin and haematocrit) and the FWEC of the third infection with *H. contortus* but no other significant correlations were found between blood cell parameters and FWEC (Supplementary Table 2, Online version only). From the work of others, however, eosinophil responses have been implicated in resistance to *T. colubriformis* (Douch et al. 1996; Windon, 1996) and in support of our findings changes in haematocrit have been correlated to *H. contortus* worm burden (Le Jambre, 1995), so we analysed these traits. Descriptive statistics for FWEC, EosDiff and HctDiff are provided in Tables 1 and 2.

Unadjusted data were checked for normality using a Shapiro-Wilk test. Mean FWEC1, 2 and 3 were cube root transformed (Cube_FWEC1, Cube_FWEC2, Cube_FWEC3) to adjust the normality properties of the data distribution. Phenotypic data were then adjusted for significant fixed effects and covariates. Fixed effects and covariates included: birth year (2003 and 2004), sex (male and female), birth weight, birth type (single and multiple), rearing type (single and multiple) and weaning weight.

From the 55 markers genotyped across the 2003 and 2004 animals of the CSIRO Gene mapping flock, 5 markers failed in the 2004 animals. Genotype data were systematically checked for genotype errors using a function in R/QTL (Broman et al. 2003) which is based on a method developed by Lincoln and Lander (1992). The error LOD provides the significance criterion and genotypes above the threshold Error LOD > 3 were removed. Map positions of markers were taken from the Australian Gene Mapping website (http://rubens.its.unimelb.edu.au/~JILLM/jill.htm).

**Linkage analysis**

QTL analyses were performed using R/QTL (Broman et al. 2003). The data were analysed using a maximum likelihood approach (Dempster et al. 1977). To explore whether results were affected by the analysis method used, additional analyses were undertaken using Haley–Knott regression (Haley and Knott, 1992) and a non-parametric approach, an extension of the Kruskal–Wallis test as described by Kruglyak and Lander (1995). All analysis approaches gave very similar results and only the maximum likelihood results are reported. Genotype probabilities were calculated at 1 cM steps. Experiment-wide significance thresholds of \( P < 0.05 \) were evaluated by permutation testing (1000 permutations). Confidence intervals were calculated as 95% Bayesian credible interval in R/QTL (Broman et al. 2003). The false discovery rate (FDR) was established by calculating the ratio of expected to observed QTL resulting from the analysis. The expected number of QTL found by chance is the product of the number of chromosomes, the number of traits and the significance threshold.

**RESULTS**

**Descriptive statistics**

Table 1 shows the descriptive statistics of FWEC on the 3 sampling days following each of the 3 infections and their means. The data demonstrate that the challenge protocol applied in this experiment resulted in high levels of individual variation within each infection.

Table 2 summarizes the mean, minimum, maximum and standard deviations of the eosinophil counts for the primary and secondary infections and haematocrit level for the tertiary infection. Eosinophil counts increased and haematocrit decreased with increasing length of parasite infection.

**Linkage analysis**

Table 3 summarizes the significant results from the linkage analysis and provides detailed information on the position of the putative QTL, adjacent markers, permutation LOD score and the 95% confidence interval. The LOD profiles are shown in Figs 1–3.

Chromosome 22 showed the most significant QTL for Cube_FWEC2. The 95% confidence interval was 23 cM and is the smallest for the putative QTL found in this study. Chromosome 21 yielded a putative

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**Table 1. Descriptive statistics of faecal worm egg counts (in eggs/gram) at 3 sampling times and their means after infection of the sheep from the CSIRO Gene Mapping flock born in 2003 and 2004 (n = 399)**

<table>
<thead>
<tr>
<th>Faecal worm egg count</th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>Stddev</th>
</tr>
</thead>
<tbody>
<tr>
<td>FWEC Day 21</td>
<td>2302</td>
<td>0</td>
<td>6300</td>
<td>1206</td>
</tr>
<tr>
<td>FWEC Day 28</td>
<td>2761</td>
<td>0</td>
<td>6700</td>
<td>1205</td>
</tr>
<tr>
<td>FWEC Day 35</td>
<td>3391</td>
<td>100</td>
<td>8200</td>
<td>1349</td>
</tr>
<tr>
<td>Mean FWEC1</td>
<td>2818</td>
<td>33</td>
<td>5600</td>
<td>981</td>
</tr>
</tbody>
</table>

**Infection 2 (*T. colubriformis*)**

| FWEC Day 21           | 2502 | 0   | 7800| 1286   |
| FWEC Day 28           | 3061 | 0   | 12800|1578    |
| FWEC Day 35           | 3777 | 0   | 10800|1673    |
| Mean FWEC2            | 3113 | 0   | 7400| 1174   |

**Infection 3 (*H. contortus*)**

| FWEC Day 21           | 6654 | 0   | 32400|4662    |
| FWEC Day 28           | 14477| 0   | 52200|9215    |
| FWEC Day 35           | 15823| 0   | 56200|8710    |
| Mean FWEC3            | 12307| 0   | 40400|6710    |
QTL for EosDiff1 at 66 cM at the end of the chromosome. Only 3 markers were genotyped on chromosome 21.

Chromosome 3 showed 2 significant QTL for Cube_FEC2 and EosDiff2 in 2 different positions on the chromosome. The 95% confidence intervals for both QTL were very large. No QTL were found for any indicator traits for the tertiary infection with *H. contortus*.

The significant putative QTL found in this study only explain between 1.36% and 3.26% of the total phenotypic variance and their effect sizes are small (Table 4). The QTL effects are expressed as the phenotypic average of the progeny carrying the Romney allele minus the phenotypic average of those carrying the Merino allele. The QTL effect for Cube_FEC2 was negative, indicating that the Romney allele was associated with lower egg counts.

Six parasite resistance traits were analysed on 8 chromosomes at a significance level of $P<0.05$ and 2+ QTL were expected to be found by chance. Four putative QTL were found. This results in a false discovery rate (FDR) of FDR = 80%.

**DISCUSSION**

This study investigated putative QTL for measures of parasite resistance using *H. contortus* and *T. colubriformis* infections in sheep. Four putative QTL for measures of parasite resistance to *T. colubriformis* in sheep were found; two on chromosome 3, and one each on chromosomes 21 and 22. Previously published studies have not reported QTL for internal parasite resistance on chromosomes 22, but Moreno *et al.* (2006) found a putative QTL for FWEC in response to a field infection on chromosome 21 and several studies have found evidence for QTL on chromosome 3. In a fine mapping study on a Romney population, a QTL was mapped close to a region that contains the interferon gamma gene on chromosome 3 (Paterson *et al.* 2001). Similarly, Coltman *et al.* (2001) confirmed an association between alleles at the interferon gamma locus and resistance indicator traits in response to a natural *Teledorsagia circumcincta* infection in Soay sheep. A more recent study by Davies *et al.* (2006) also found strong evidence for parasite resistance QTL on chromosome 3. Beh *et al.* (2001) reported a putative QTL on chromosome 3 for cube root transformed mean FWEC of a secondary infection with *T. colubriformis*. Although, Beh *et al.* (2001) pointed out that the marker distances were large in their study for this particular region. The conjunction of findings between our study and others on chromosome 3 suggests strongly that future work on this chromosome is warranted. Crawford *et al.* (2006) detected 6 QTL for parasite burden and immune response to infection in a large outcross design on chromosomes 2, 8, 11 and 23. None of these results overlap for the 3 chromosomes that were genotyped across both studies, but the phenotypes recorded were different.

The QTL described herein have large confidence intervals and account for a small percentage of the total phenotypic variation of the traits. The expression of a particular level of resistance to internal parasites is the consequence of a cascade of events that underlie the control of a large number of genes, as shown in a number of recent gene expression studies (Diez-Tascon *et al.* 2005; Andronicos *et al.* 2009). This study found QTL with much smaller effect sizes probably because a large number of genes control a complex trait like parasite resistance; a hypothesis that is shared by Crawford *et al.* (2006). It is also supported by findings of Marshall *et al.* (2009), who found a large number of QTL with moderate size effects in a mapping experiment for resistance to *H. contortus*. A simulation study by Hayes and Goddard (2001) has shown that the majority of putative QTL for any trait are expected to have a small to moderate size effect. If internal parasite resistance is truly controlled by a large number of genes with small effect, then this study might not have been powerful enough to detect the small effect sizes, which could explain the lack of significant QTL for internal parasite resistance in this study.

Our study shows the non-alignment of QTL for FWEC following *T. colubriformis* infection and the change in eosinophil numbers as a result of these infections for this particular experiment. Also there was no evidence of a phenotypic relationship between the two traits (Supplementary Table 3, Online...
version only) even though Davies et al. (2005) found strong negative genetic and moderately negative phenotypic correlations between FWEC and eosinophil count in Scottish Black face sheep. Earlier research on the relationship between eosinophil numbers and FWEC has also resulted in conflicting results. Windon et al. (1987) observed an increase in eosinophil responsiveness as characteristic of parasite resistance, whereas Woolaston et al. (1996) observed no relationship between FWEC and eosinophil count.

A novel aspect of the experimental protocol used in this study was that the sheep were artificially challenged with 2 parasites in separate infections. This is a different approach from that used in other studies in which resistance of sheep to a single parasite (Beh et al. 2001; Marshall et al. 2005) or the responses to mixed field infections (Paterson et al. 2001; Davies et al. 2006) were investigated. In the most recent major mapping study on parasite resistance

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr</th>
<th>LOD score</th>
<th>Perm LOD</th>
<th>Position (cM)</th>
<th>Marker bracket</th>
<th>95% CI (cM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cube_FWEC1</td>
<td>3</td>
<td>1.93</td>
<td>1.83</td>
<td>62</td>
<td>OARCP34 CSAP31e</td>
<td>278-4</td>
</tr>
<tr>
<td>EosDiff2</td>
<td>3</td>
<td>2.20</td>
<td>1.77</td>
<td>213</td>
<td>CSAP019 MAF23</td>
<td>182-0</td>
</tr>
<tr>
<td>EosDiff1</td>
<td>21</td>
<td>1.80</td>
<td>1.22</td>
<td>66</td>
<td>CSRD272 BMS1948</td>
<td>33-4</td>
</tr>
<tr>
<td>Cube_FWEC2</td>
<td>22</td>
<td>3.03</td>
<td>1.24</td>
<td>52</td>
<td>HEL11 BM6041</td>
<td>23-0</td>
</tr>
</tbody>
</table>

Table 3. QTL position, marker bracket and 95% confidence interval (95% CI) of significant QTL as assessed by the 5% permutation LOD score (PermLOD) for the parasite resistance traits.
Crawford et al. (2006) lambs were subjected to consecutive field challenges, in a manner relevant to commercial sheep production. However, the artificial challenges utilized in this study enabled the investigation of resistance traits to specific Strongylid nematodes (H. contortus and T. colubriformis) separately.

No overlapping QTL were found for primary and secondary infections with T. colubriformis. One reason might be that around 50% of lambs in this study carried a parasite burden prior to the first infection, because it is difficult to avoid an infection of lambs under field conditions. We minimized exposure by using anthelmintic treatment of the dams and by paddock rotation, and also by beginning the experiment as soon as possible after weaning the animals, but there was still a low level of exposure to infection. Consequently, the first infection with T. colubriformis was not a primary infection for at least half the animals, although it was a synchronized infection given after resident parasites had been removed using anthelmintic drugs.

Our approach enabled the investigation of underlying resistance pathways to only one or both parasites in series. It has been shown that selection of resistance to one or other of these parasite species leads to cross-resistance to both parasites (Ingham et al. 2007). In this study, no significant results were found that would suggest overlapping putative QTL for resistance to H. contortus and T. colubriformis infection. Nevertheless, genetic relationships between FWEC from infections with one species of nematode and FWEC from infections with other species has been demonstrated (Windon et al. 1987; Gruner et al. 2004) and might be attributable to loci that could not be detected in this study.

The extent of haematological phenotypes collected has not been reported in previous mapping studies for internal parasite resistance in sheep. The combination of measurement of FWEC and blood cell parameters enables the possibility of gaining insight into the genetic relationships between egg counts taken at different infection times and changes in the numbers of circulating blood cells. This first analysis has concentrated upon the changes in cell numbers between day 0 and day 28 of infections, and finds no common chromosomal regions associated with FWEC and haematological measures. However, this does not exclude the possibility of QTL influencing both these traits in other populations.

This study detected a low number of putative QTL. Due to constraints, data were only available for 1 single sire half-sib family with 399 progeny. Potentially this could have been another reason for the small number of significant QTL found, if this particular sire did not segregate for QTL of detectable size even though the design provides sufficient power for the detection of moderate size QTL. The validity of the results of this study is limited to this particular sire family and can not be generalized. Furthermore, it could be possible that the Romney and Merino breeds were genetically not as divergent as expected from phenotypic observations (Windon et al. 1993). Further work needs to investigate and verify the results in multiple families with higher marker density.

**ACKNOWLEDGEMENTS**

The CSIRO Romney × Merino Gene Mapping flock was conceived by Dr Ian Franklin, and the F1 ram that sired the animals generated for this study was produced as part of his earlier work with this flock. Ken Beh and Ross Windon contributed to the design of the parasite infection protocol used in the study and Jill Maddox has assisted with advice on microsatellite markers. We also gratefully acknowledge the technical assistance of Heather Brewer, Jody McNally, Callum Mack, David Callan, Brad Hine, David Paull, Brian Dennison, Jen Smith, Grant Uphill, Judi Kenny and Greg Good.

<table>
<thead>
<tr>
<th>Trait</th>
<th>%Var</th>
<th>QTL effect</th>
<th>S.E.</th>
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<tr>
<td>Cube_FWEC1</td>
<td>1.36</td>
<td>2.534</td>
<td>1.151</td>
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<tr>
<td>EosDiff1</td>
<td>2.06</td>
<td>0.023</td>
<td>0.008</td>
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<tr>
<td>Cube_FWEC2</td>
<td>3.26</td>
<td>-3.636</td>
<td>1.031</td>
</tr>
</tbody>
</table>

Fig. 3. LOD profiles of chromosome 21 for EosDiff1, indicating the significant QTL by the vertical broken line, the permutation threshold (PermLOD) by the horizontal broken line and the confidence interval (95% CI).
REFERENCES


