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GENETIC AND BIOLOGICAL APPROACHES TO MODULATE NEMATODE RESISTANCE MECHANISMS IN SHEEP

D.L. Emery and K.J. Beh
Faculty of Veterinary Science, University of Sydney, Sydney, NSW. 2006, and
MLA-AWI Sheep Genomics Program

SUMMARY
An integrated approach using mapping and functional biology to discover and apply large effect QTL and gene expression differences underlying worm resistance in sheep is presented and discussed.
Key words: sheep, genes, nematode resistance, immunology

INTRODUCTION
Widespread development of anthelmintic resistance amongst economically-important gastrointestinal nematode (GIN) parasites of ruminants awakened interest in the use of genetic selection of resistant animals or other means to enhance host-protective responses for worm control. Estimates of heritability of faecal egg count (FEC) range from 0.23-0.41 (Behnke *et al*., 2003a; Dominik, 2005) and has been exploited to make positive gains in resistance in traditional selection programs (Albers *et al*, 1987) . Phenotypic selection requires expression of resistance genes so progress would be enhanced using marker-assisted selection. Studies designed to find resistance markers have been reviewed recently (Dominik 2005).

However worm resistance assessed by FEC is likely to be influenced by many alleles at different loci, since it is a trait with a complex immunological basis. Mechanistic studies in selected families and resource flocks with contrasting GIN resistance phenotypes have identified broadly the nature of acquired responses central to protective immunity (Beh *et al*., 2002; Behnke *et al*., 2003a). Immunological dissection of resistance components in outbred populations is difficult, so historical results analyzing resistance mechanisms are not easy to interpret and are complicated by diverse infection/challenge models, varying parasite doses, distinct modes of infection, different sheep breeds and the range of phenotypic measures. A second level of complexity is the interrelationship between resistance as defined by effects on the GIN, and "resilience" defined as the ability to maintain production and reproduction during ongoing parasite challenge or to minimize the pathophysiological effects of parasitism. So resistance to infection may not necessarily share the same fundamental mechanisms as disease resistance (Bisset and Morris, 1996), although nutrition may underscore both (Kahn *et al*., 2000).

Recently, the advent of high throughput molecular techniques and methods to monitor and manipulate host-parasite relationships in vitro and in vivo, have allowed construction of a synergistic integrated genetic and immunological/parasitological approaches for gene discovery. This paper examines the principles involved in combining various genomic technologies into an integrated pipeline for discovery, characterization and exploitation of GIN resistance genes benefiting the sheep industry.

GENETIC APPROACHES

Historically, technology has dictated approaches to genome analysis (marker development) so early studies sought associations with the few polymorphic genetic systems available such as haemoglobin type and the major histocompatibility complex (MHC) (see Beh *et al.,* 2002). More recent candidate gene studies reported significant associations with IgE (*T. colubriformis*) and the gamma-interferon gene (IFN-γ) (*Telodosagia circumcincta*) the latter apparently confirming results from an earlier limited whole genome scan (see Beh *et al*., 2002). Associations were also reported for DRB MHC alleles responsible for 58-fold FEC reductions after natural infections with *Te.circumcincta* (see Beh *et al*., 2002; Dominik, 2005). Subsequently, availability of genome-wide microsatellite markers revealed using PCR has enabled scans for markers using whole genome linkage analysis (Beh *et al*. 2002; Raadsma *et al*., 2005). Scans were carried out in a number of resource flocks using various parasites and, although most remain unpublished, anecdotally many putative QTL have been identified with those on ovine chromosomes 1, 3, 6 and 20 the most promising (see Table II in Dominik, 2005). Considerable effort has gone into performing these scans, and clearly one way to maximize the benefit from them is to undertake iterative meta-analyses designed to verify QTL across differing resource flocks. These analyses should be supplemented with ongoing studies to provide additional data and further linkage analyses to confirm and refine effect size and position of putative QTL. Supplementary studies in commercial sheep based on LAMBPLAN® data (http://www.mla.com.au/lambplan/) would assist industry adoption of relevant findings. The sheep genomics (SG) initiative has developed a large-scale fine-mapping flock at Falkiner Memorial Field Station using industry-relevant sires to increase the number of progeny for detection of QTLs with greater certainty (Oddy et al., 2005). A comprehensive range of phenotypic measures is being taken to include traits associated with GIN resistance and resilience.

While confirmed QTL for difficult to measure traits such as parasite resistance are potentially of use in selection programs, the ultimate aim of most genetic studies is to identify the actual alleles responsible for the QTL effect. Definition of QTL architecture then presents the possibility of devising novel non-genetic means for parasite control based on directed manipulation of specific causal alleles. However serious problems arise for genetic analyses in attempting to fine map QTL in domestic animal populations. Problematic factors include the lack of dense marker maps based on single nucleotide polymorphisms (SNPs) and the impracticality of breeding and phenotyping the deep or broad population structures required for such fine scale mapping. While intensive SNP development is occurring in cattle, a similar exercise in sheep is worthy of investment. These factors are further compounded by the operation of linkage disequilibrium over considerable distances in domestic sheep populations (McRae *et al*., 2002), limiting fine mapping resolution. These constraints mean that parasite resistance QTL will be difficult to map beyond 5-10cM resolution.

Another aspect of worm resistance complicating complete QTL dissection is the complexity of the faecal egg count. This is a complex trait representing the net outcome of an intricate series of interactions between host and parasite (Dobson *et al*., 1990). It will be necessary to understand the interwoven network of resistance gene expression pathways operating during disease pathogenesis that result ultimately in acquisition of immunity.

FUNCTIONAL ANALYSIS OF RESISTANCE GENES AND PATHWAYS

The slow development and expression of GIN resistance mechanisms in the abomasum and intestine occurs progressively over several months following specific recognition of parasite antigens. The kinetics of acquired immunity against *T. colubriformis* is expressed as inhibition of larval establishment after 5-7 weeks of continuous infection, reduction in female fecundity after 12-16 weeks and rejection of established adult worms after 20 weeks (Dobson *et al*., 1990; Emery *et al.*, 1993). While the induction phase of acquired immunity is worm-specific and generates allergic-type immune reactions, the inflammatory effector response is generally non-specific and can affect heterologous nematodes, engaging inflammatory and neuronal cells and mediators, as well as enteric smooth muscle (Behnke *et al*., 2003b; Emery *et al*., 1993).

Identification of genes involved in mediating GIN resistance involves both gene discovery and candidate gene approaches, where an essential prerequisite is identification of the key times and tissues in which different levels of resistance is expressed in animals of contrasting phenotype. The candidate gene approach using molecular quantitation by the polymerase chain reaction (PCR), of mRNA from tissue is predicated on functional biology and historical data implicating immune response genes coding for recognition molecules, receptors and cellular products involved in allergic T-helper 2-type (TH2) responses as well as genes active in enteric repair and locomotion. It is a "short-cut" to outcome, but risks not finding the "real" gene or missing important genes.

Based on availability and validation of homologous and heterologous microarrays for sheep (Tao *et al*., 2004), a global gene expression approach has been used recently. Using microarrays on Perendale sheep, gene ontologies involved in immune responses and enteric smooth muscle have been implicated in worm immunity (Diez-Tascon *et al.,* 2004). However, the integration of physical and functional mapping has substantially greater potential to identify key resistance genes than either method independently by enabling a gene discovery model that makes no assumptions about putative genes or functional pathways involved in parasite resistance.

COMPLEMENTATION OF GENETIC AND FUNCTIONAL APPROACHES

To utilize fully the power of the integrated gene discovery pathway, the widest possible phenotypic and genetic contrasts are required in the starting animal resources to optimize chances of discovery of large effect QTL and to maximize gene expression differences. Critical expression profiling is done sequentially during larval establishment, worm growth and reproduction and adult worm killing. The combined fine-mapping and gene expression approaches overlaps positional information of QTL with differential gene expression and has the potential to filter and reduce the number of potential candidate genes. Such positional candidates are assumed to be responsible for the primary gene expression event resulting in acquisition of worm resistance or are differentially expressed during the initiation of the protective immune response. Even so, this task will not be straightforward.

A key output of microarray analyses could be identification of "advanced" phenotypes based on specific gene expression patterns. Advanced phenotypes would be used in further QTL scans on the basis that expression of members of a gene pathway affected by a given QTL will map to the location of the QTL, thus revealing both. A potential limitation is the inaccessibility of most mucosal tissues

to almost all except experimental studies, so it would be an added challenge to detect these phenotypes based on gene expression changes in blood.

CONFIRMATION AND MODULATION OF GENE ACTION

Isolation of a manageable number of differentially expressed genes represents the first step in a gene discovery pipeline. Functional analysis of selected candidate genes, characterising their role in parasite susceptibility pathways is the next step. Medium/high throughput screening assays will be used where gene action can be manipulated in primary cell cultures. Readouts from these systems include cellular bioassays and immunoassays for host cell products, including cytokines, pharmacological mediators and hormones. Direct parasite readouts used in conjunction with cell transfection assays will include egg hatch and larval development and migration assays. Other assays might include effects on *Haemonchus contortus* tissue cell lines (Coyne and Brake, 2001) and biochemical and gene expression readouts to detect subtle damage to the viability of infective larvae exposed to candidate gene products. Such nematode parasite assays could advantageously draw on genomic manipulation techniques developed for *C. elegans*, and the available libraries of *C. elegans* gene-deficient mutants. Finally, complete functional analysis of 3-5 key candidate genes with major effects on parasite infection will involve further intensive studies in *in vivo* and *in vitro* experiments in animal models such as transgenic mice knockout or over expression models.

The complete functional analysis of key genes affecting worm resistance will form the basis for commercially attractive innovative products for parasite control. These may be based either on direct modulation of gene function or on manipulation of the effect of protein products to produce novel interventions, therapeutics or bioactives.

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