AGMARDT Agribusiness Innovation Grant A16027

New Approaches to Control Parasites; Final Report

May 2107

- **Executive Summary**

For New Zealand to meet the Government's target of doubling agricultural exports by 2025, new technologies to control infectious diseases will be necessary. Parasite infections are the most important communicable, infectious disease in NZ ruminants. They are poorly understood. Therapeutic control has changed little since the development of anthelminthic drenches 40 years ago and diagnosis is also largely unchanged, relying on manual faecal egg and larval counts to provide an estimate of worm burdens as an indicator of disease status.

The research aims of this project were twofold. Firstly, when an animal is scouring or losing weight we wanted to be able to better diagnose why. To this end we sought to augment our (existing) Johne's disease faecal test technology to simultaneously detect endoparasite species commonly affecting red deer. The goal was to develop a composite diagnostic test for animals presenting with generalised, nonspecific clinical indicators of enteric disease such as scouring or progressive weight loss. Distinguishing between Johne's and parasitic disease remains a continuing challenge in disease control where accurate, disease-specific diagnosis will expedite properly informed and appropriately targeted treatment and management.

Secondly, we wanted to develop better ways to measure the natural resistance or susceptibility to parasitic infection evident in livestock populations that it may be exploited. To do this we employed methods we have developed to monitor ruminant (cervine) immune responses to Johne's disease to measure immune responses to parasitic infection. Improved diagnostics would allow us to identify infected, susceptible animals or resilient and resistant animals with protective immunity for further study. The immunological outcomes that give rise to these states are poorly understood and are a major knowledge gap that limits management of parasitic infection in domestic livestock. These studies were exploratory and a prelude to future research to focus on superior immunodiagnostics to identify susceptible animals for targeted anthelminthic treatment and to identify markers of immune protection to improve future vaccine development and support genetic

selection of animals with superior resistance or resilience to parasites. Data derived from a study of one species of ruminants will likely translate to different ruminant species allowing for improved parasite management systems across the primary sector.

- **Stated Goals of the Project -**

Diagnostics. With technology and knowhow already in place to routinely and quantitatively assay ruminant faecal samples for bacterial DNA shedding (Johne's) we wished to turn our attention to egg and larval (L1-L4) stages of those parasitic infections most relevant to primary industry [initially Ostertagia spp. (gutworm) and Dictyocaulus spp. (lungworm)]. Informed by similar studies in the literature, we sought to develop rapid DNA-based qPCR tests for parasite eggs and larvae shed in the dung of affected hosts as an alternative to conventional manual faecal egg counting and as an adjunct to our routine Johne's testing.

We believe that a significant point-of-difference between the service DRL are able to provide and similar efforts ongoing internationally is that DRL already have in place robust and semi-automated systems for efficient and economical processing of ruminant faecal samples for high-quality DNA recovery and for the express purpose of DNA based diagnostics (due in no small part due to a previous AgMardt Agribusiness Innovation Grant A15006, 2014), through which we have developed and brought to market a high-throughput faecal shedding diagnostic platform to stratify bacterial shedding in Johne's disease affected animals (deer, cattle, sheep, goats and alpaca). Often the most difficult aspect of progressing the proposed assays through to routine application is the translation from a complex, expensive and low-throughput research application limited to the laboratory bench into a practical, inexpensive and routine diagnostic tool for farm use. In the case of this project, this most challenging aspect has already been accomplished at DRL as our laboratory routinely extracts microbial DNA from faecal material from scouring deer submitted for Johne's disease diagnosis. As the greatest single cost-component of the Johne's test lies in the DNA recovery step, a secondary assay for the presence of parasite DNA within the same extracted sample may be conducted at minimal additional cost, thereby adding value for the client.

Immune responses to parasites. The basic methodologies for assaying cervine immune responses to parasitic challenge were already in place through our Johne's

work. We have recently completed a 5 year research programme with DeeResearch and Callaghan Innovation designed to identify by laboratory assay immune gene expression markers characteristic of deer with extreme phenotypes for Susceptibility (S) or Resilience (R) to Johne's disease. The approach we have adopted involves measurement of the expression levels of a series of candidate gene targets (identified both through the literature and as a result RNA-Seq transcriptomics investigations performed previously by DRL) in blood cells from animals with S or R phenotypes as revealed by experimental challenge. Measurement of the level of specific mRNA produced in peripheral blood cells is taken as a proxy for the level of the protein biomarker produced by that gene.

The purpose of this work has been to identify biomarkers whose expression levels are associated with, and predictive for, either the S or R phenotype by laboratory assay. For this project, the immune profiles of resistant adults which display protective immunity (low worm burden in a challenge environment) were compared with responses seen in unprotected, susceptible juveniles (high worm burden) and the responses of *ex vivo*, antigen-stimulated immune cells will be assayed using panels of gene expression assays developed as part of our Johne's research programme. Our previous studies involving bacterial infections have identified key pathways of immunity and gene markers associated with protective immunity or disease and this same approach will be used to compare resilience and susceptibility to parasite infection. In general, adult ruminants if well fed and in good condition develop a natural, protective immunity to parasitic infection over time. In contrast, younger stock are more susceptible to parasites and require repeated cycles of anthelminthic treatment as juveniles to control disease. Because the protective response in adults is poorly understood it is necessary to chart pathways of immunity more definitively so that the immune response in juveniles can be reprogrammed to develop protective immunity much earlier. This would preclude the need for repeated anthelminthic treatments in young animals, limiting selection for parasites with increased resistance to currently used anthelminthics.

The present project sought to develop direct DNA tests for gastrointestinal parasite species from ruminant dung samples submitted for Johne's disease testing and to use these methods to augment test results and provide added value diagnostic information to farmers and veterinarians. The project sought also to apply technology developed to differentiate host resilience from susceptibility to Johne's

disease to initiate a research programme which would apply similar approaches to parasitism.

- **Outcomes Achieved by the Project**

As a direct result of AgMardt support received, we now have in place DNA based diagnostic assays for gut parasites (Ostertagia spp.) and bovine and cervine lungworm (Dictyocaulus spp.). Having demonstrated necessary proof of principle of a DNA based diagnostic for parasites shed in faeces, we are now working towards calibration of the assay against conventional methodologies (faecal egg and larval counts) using a cohort of young red deer exposed to naturally acquired parasite infection and monitored routinely at the AgResearch Invermay agricultural campus. The disease status of each of these animals will be confirmed retrospectively, following necropsy of animals experimentally infected with Ostertagia parasites

- **Detail results achieved against the Milestones set out in the original application**

Milestones 1-3 (from AGMARDT Progress Report for period ending 11/16)

Milestone Number	**Milestone** ***(As per AGMARDT agreed schedule)***	**Completion Date**		**Percent Complete**
		Original	**Est. or Actual**	
1	Design and test qPCR assays for nominated endoparasite species. Assess current inhouse faecal processing procedures for DNA recovery from mycobacteria and optimise to capture and enrich for endoparasite eggs and larvae.	04/16	08/16	100%
2	Monitor cytokine (immune hormone) gene expression levels in peripheral blood from parasitised adult and juvenile deer using specific parasite antigens or polyclonal (non-specific) activators.	08/16	11/16	100%
3	Validate and finesse diagnostic methods to the point of routine application. Advertise new value added service to clients and promote through appropriate media, DRL website, promotional material, field days etc...	12/16	03/17	100%

Milestone 1.

DNA assays directed against conserved strongylid worm sequences based upon an internal transcribed spacer (ITS-2) region of ribosomal DNA of nematodes[1] were custom synthesised and tested against two nominated parasite species *Ostertargia spp.* (gutworm) and *Dictyocaulus spp*. (lungworm). By utilising public sequence databases, coupled with sequence comparisons already published within the literature, we prepared quantitative PCR assays for both of these species. To test their performance, we received samples of adult *Ostertagia leptospicularis* from consultant parasitologist Dr Paul Mason of Christchurch and samples of adult *Dictyocaulus eckerti* cervine lungworm from AgResearch Invermay. Genomic DNA prepared from these specimens was used to confirm PCR amplification was successful.

To further assess performance from clinical material, archived DNA samples which originated from faecal samples submitted to DRL for Johne's disease testing from clinically presenting deer (scouring coupled with progressive weight loss), but which were subsequently shown to be negative for Johne's, were tested successfully and shown to be positive for parasite DNA. Finally, a mob of 48 weaner deer from which associated Faecal Egg Count (FEC; Ostertagia) and Faecal Larval Count (FLC; Dictyocaulus) data were available were coassayed with the newly developed DNA test, revealing compelling correlation (Fig. 1, below).

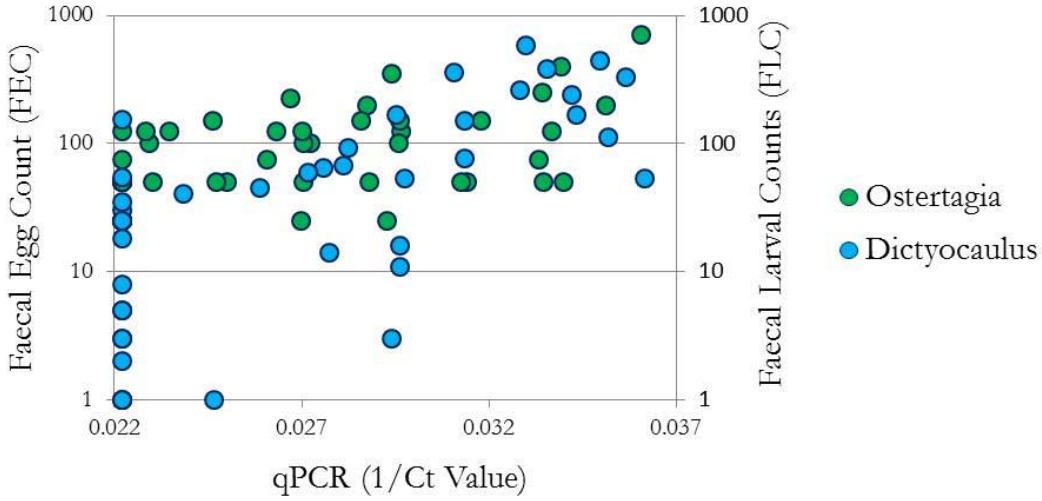

Figure 1. Faecal egg (Ostertagia) and larval (Dictyocaulus) counts plotted relative toPCR results.

[1] Bisset SA, Knight JS, Bouchet CL: A multiplex PCR-based method to identify strongylid parasite larvae recovered from ovine faecal cultures and/or pasture samples. Veterinary parasitology 2014, 200(1-2):117-127.

Milestone 2.

Leading on from the successful development of DNA based parasite diagnostic tools under Milestone 1 we attempted to assay some host immune parameters in response to parasitic infection using a panel of five gene expression markers (Interferon gamma (IFNγ), Interleukin-1α (IL-A), Interleukin-1β (IL-1B), Interleukin-2 (IL-2) and Interleukin-17 (IL-17)) which were developed to differentiate animals exhibiting polarised phenotypes for resilience or susceptibility to Johne's disease (Jd). The animals involved are part of an ongoing Jd experimental infection trial and were simultaneously exposed naturally to parasitic challenge in the field.

In early September, this cohort of 37 mixed sex, yearling deer were faecal sampled as part of routine monitoring for mycobacterial shedding and coassayed for strongylid endoparasite DNA signatures (e.g. *Ostertagia* spp.). Fourteen of the thirty seven animals showed detectable levels of strongylid DNA in their faeces at this time. One month later, 11 of the 37 animals tested positive for stongylid DNA; 5 of these animals were resampled the following week and were tested both for parasite DNA and for faecal egg counts by conventional methodology. Three of the 5 animals remained positive for parasite DNA while the remaining two were negative. The same 3 animals which tested positive for parasite DNA were also positive for faecal egg counts although, at the low end of the scale, and the samples which were negative for DNA were also negative for faecal egg counts. Following routine drenching the following week all animals became negative for both DNA and faecal egg counts.

To quantify host immune cell responses to polyclonal stimulation, mononuclear cells were harvested from peripheral whole blood obtained by jugular venepuncture into standard 10ml vacutainer tubes and stimulated in vitro using staphylococcal enterotoxin B (SEB) to drive the differential expression of target transcripts. Total RNA was recovered following cellular lysis and cDNA generated by reverse transcription. Copy numbers of the biomarkers were determined by quantitative PCR using SYBR Green chemistry coupled with gene specific primers and relative expression determined using $\Delta\Delta$Ct values following normalisation to a housekeeping gene and expressed as fold change of the stimulated sample relative to unstimulated control cells. Gene expression signatures from animals exhibiting evidence of parasite infection (n=5) were compared to those obtained from animals exhibiting no evidence of parasite infection (n=32) and the results are presented here in Figure 2.

In contrast to Jd where gene expression datasets derived from phenotypically resilient and susceptible animals are observed to exhibit spatial separation when the data contributed by each biomarker are superimposed on top of one another, there was no obvious separation of parasitised vs non-parasitised gene expression signatures in this instance. This could be due to parasite evoked immune responses being masked by the developing Jd infection or the comparatively mild nature of the field-acquired parasitic challenge coupled with the small numbers of animals testing positive for parasites. Additionally, it may be that this panel of expression targets which were designed to monitor Th1 (cellular) immunity to intracellular pathogens (Johne's disease) are inappropriate for Th2 (humoral) immune responses elicited by extracellular pathogens such as parasites. Future studies are planned to evaluate expression of genes associated with Th2 activation. This pathway of immunity is characterised by cytokines (IL-4, IL-5, IL-10, IL-12, TGF-β and SOCS-1) that evoke antibody (IgG_1, IgA & IgE) associated with protective immunity to parasites. The initial gene expression results demonstrate proof of principle for the concept of a composite DNA diagnostic for Johne's disease and intestinal parasites in farmed deer going forward.

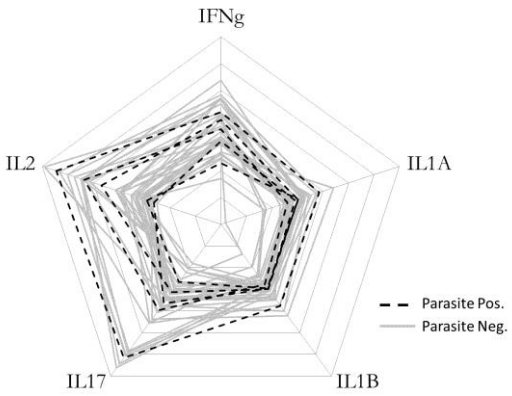

Fig. 2. Radial plot summarising gene expression responses of immune cells from animals testing positive (n=5) and negative (n=32) for strongylid parasite DNA in dung samples.

Milestone 3.

We are now at a point where we can routinely assay for *Ostertagia spp*. and *Dictyocaulus spp*. in addition to *Mycobacterium avium* subsp. *paratuberculosis* (the causative agent of Johne's disease) in client submitted dung samples. A January post on our fledgling Facebook page[2] highlighted the additional availability of this service as part of our diagnostic repertoire at DRL and acknowledged the support of AgMardt in this endeavour. More recently, DRL attended South Island Field Days at Kirwee in association with Ag@Otago and used the opportunity to promote our diagnostic services, including parasite testing alongside Johne's disease. We will also be attending national field days at Mystery Creek in June and look forward to showcasing this new technology at that event also.

[2] https://www.facebook.com/DRLOtago/

In two pages report on how the project enabled the applicant to:

- transform a business or industry;
- speed up innovation and respond quickly to opportunities arising from the marketplace; and
- facilitated linkages between innovative New Zealand researchers, producers, processors and marketers.

The immediate benefit to the NZ farming community is the availability of a novel diagnostic test for parasite infection *Map* shedding in poorly performing livestock, with quicker result turnaround than is currently available using existing FEC technology. Further work is required to properly calibrate the resulting data to maximise the value of the quantitative information returned by the test.

- Identify all additional achievements and/or provide additional comment;

A major limiting factor in the study of improved management is that there is little understanding of the development of protective immunity or disease related immune pathology in parasitised animals. Serious questions have been raised as to the validity of faecal egg counts as indicators of disease. Alternative molecular technology developed in the current project should shed new light on parasitic disease phenotypes (linked to susceptibility), and identify pathways of protective immunity seen in animals that are resilient to disease. The logical extension of these studies would be to develop futuristic management strategies that enhance early development of protective immunity in young animals. This would free farmers from the tyranny of repeated (28 day) cycles of antihelminthic treatment in juveniles, during the most rapidly growing phase of animal production. Restricting the need for antihelminthics treatment would offset the emergence of drug resistance in parasites, an associated consequence of repeated use of antiparasite therapy.

- A summary of the project is to be provided for media release (1 page)

For New Zealand to meet the Government's target of doubling agricultural exports by 2025, new technologies to control infectious diseases are needed. Parasitic infections are the most important infectious disease in NZ ruminants. They are poorly understood and therapeutic control has changed little since the development of anthelminthic drenches 40 years ago. Diagnosis remains largely

unchanged, relying on manually performed faecal egg and larval counts to estimate degree of worm burden. The research aims of the current study were twofold; firstly when an animal is scouring or losing weight we wanted to be able to better diagnose why. To this end we have augmented our (existing) Johne's disease faecal test technology to concurrently detect endoparasite species commonly affecting red deer. The goal has been to develop a composite diagnostic test for animals presenting with generalised, nonspecific clinical indicators of enteric disease such as scouring or progressive weight loss. Distinguishing between Johne's and parasitic disease remains a continuing challenge in disease control and early differentiation will expedite informed and appropriately targeted treatment and management. With this assistance from AgMardt we have developed rapid, quantitative and species-specific DNA tests for parasite eggs and larvae shed in the dung of affected hosts as an alternative to conventional faecal egg counts and as an adjunct to routine Johne's testing.

Secondly, we have begun to investigate the emergence of natural resistance or susceptibility to parasitic infection evident in naturally exposed livestock populations. To this end we have utilised methods we developed to monitor ruminant (cervine) immune responses to Johne's disease to monitor immune responses to parasitic infection. Improved diagnostics will allow us to differentiate between diseased, susceptible animals, and resilient/resistant animals with protective immunity for further study. These studies are exploratory and a logical prelude to future research to develop immunodiagnostics that identify diseased/susceptible animals for targeted anthelminthic treatment. By contrast the identification of markers of immune protection/resilience will inform future vaccine development and support genetic selection of animals with superior resistance or resilience to parasites.

Parasitism ranks as the most costly type of infectious disease affecting NZ livestock, with losses >$700M pa. Currently the strategy to control parasitic disease involves repeated drenching of young animals with anthelminthics using protocols that have changed little in 40 years. Repeat drenching is expensive, produces chemical residues and results in the development of drug resistance, limiting long term utility. In a world of ever increasing parasite resistance to chemical drenches, with consumer opinion signalling disapproval of resultant residues and with ever tighter margins on production systems, it is time to consider alternative approaches to the problem of parasitism. This work addresses key issues that could enhance the

production of residue-free food products in an ever discriminating International marketplace.

- A summary of total income and expenditure of the project compared with the original budget as well as copies of invoices exceeding $1,000 attached.

  Summary of expenditure:

  Total income: $92,000

  Expenditure:

Item	**Budgeted amount** *(excl. GST)*	**Actual amount** *(excl. GST)*
Laboratory costs	$60,000	$60,000
Husbandry	$32,000	$32,000
Total	**$92,000**	**$92,000**

Finally, DRL would like to express our thanks to the AgMardt trust for supporting this project. The developments described here would not have been possible without this financial assistance.