# final report

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## Quantitation of sheep nematode parasites in faeces using a PCR technique

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# Contents

1. Final Report Summary ..... 3
1.1. Achievements ..... 3
1.2. Further Development Potential ..... 3
2. Milestone ..... 4
3. Abstract ..... 4
4. Project objectives ..... 5
5. Success in achieving milestone ..... 5
5.1. Multiplexing ..... 5
6. References ..... 14
7. Recommendations ..... 14

# 1. Final Report Summary

## 1.1. Achievements

1. A flexible methodology for the specific diagnosis of strongylid infections of sheep has been developed. A multiplex test has been developed which can simultaneously detect and quantitate barber's pole, black scour and small brown stomach worms of sheep. In addition, seven single-species tests have been devised which in SYBR green-based assays can detect these three groups of worms and additional species found in sheep in Australia.
2. A simplified methodology for extracting high quality DNA from nematode parasite eggs after separation from faeces has been developed. This method has been tested using field samples and provided species composition data to complement worm egg counts (FWEC) in these samples. It is ready for commercial evaluation in its current form.
3. A feasibility study has been completed to evaluate the direct extraction of DNA from faeces and undertake PCR to replace visual FWEC and the specific identification and delineation of nematodes. Results are encouraging, showing the potential for inclusion of internal quality controls, aiding the qualitative and quantitative evaluation of nematode parasites in field samples.

## 1.2. Further Development Potential

1. The PCR methodology developed as part of this project could be extended to bacterial or viral pathogens. It would seem logical to develop a panel of PCR tests appropriate for each livestock industry and climatic zone. These tests would be marketable and faster than current techniques.
2. The project supported the evaluation of a number of existing techniques to extract DNA from faeces. We undertook additional work to improve one of these methods, but further improvements to the DNA extraction procedures would be beneficial, and perhaps necessary for full commercial application of direct faeces tests. There is scope for improvement as the focus of much international research is currently aimed at improving DNA extraction methodology. We have presented evidence that incomplete digestion of nematode eggs during the extraction procedure used by CSIRO can be responsible for the limited ability of faeces-direct methodology to deliver quantitative data.
3. The inclusion of an internal positive control is important for quality control of PCR tests. These controls enable a true negative result to be distinguished from a PCR which has failed for technical reasons. We have successfully utilised yeast spiked directly into faeces as a qualitative internal control in the completed work. The control should be further modified to enable fine quantitative adjustment in addition to the qualitative assessment of results.
4. Larval differentiation (LD) is often not possible because of low egg counts, and we have observed much variation between microscopists in commercial laboratories when presented with identical samples. LD is an unreliable technique which needs replacement with an alternative such as PCR. A formal assessment of the performance of commercial microscopy operators against PCR (using highly purified DNA from cultured larvae) would be informative and useful.

## 2. Milestone

Improve the semi-quantitative tests for the key sheep parasite species. Improvements will include:

a. Enabling the multiplexing of reactions to increase throughput and reduce costs of diagnosis using the RT-PCR (qPCR) methodology.
b. Improvement of the quantitation methodology to minimise the number of control reactions necessary per analysis.

Undertake an assessment of the feasibility of testing for drug resistance alleles in nematode populations using a faecal DNA-based PCR.

## 3. Abstract

We have developed some primers and probe sets to multiplex PCR reactions, enabling the simultaneous detection of nematode eggs from the genera *Haemonchus*, *Teladorsagia* and *Trichostrongylus*. The yeast (*Saccharomyces cerevisiae*) internal positive control is currently best amplified in a second reaction. Quantitation is possible through comparison with standard curves. When eggs or larvae are used to make DNA, these assays are robust, relatively cheap and the yeast internal control allows the detection of false negatives which arise due to contaminants. With continued testing, it may be possible to develop protocols which do not require standard curves for quantitation; however, we have not been able to dispense with standard curves at the end of the project.

Using *avr-14*, a gene potentially implicated in macrocyclic lactone resistance, we have shown that single copy genes can be amplified from DNA derived from eggs, larvae or adult nematodes, and that size discrimination can be used to distinguish alleles in a subsequent capillary electrophoresis step. High resolution melt curve analysis could also be used to distinguish alleles with differing nucleotide composition. There is good scope for the use of real time PCR to identify the presence of resistance alleles in samples of nematodes.

The direct extraction of DNA from whole faeces is an important goal, as it would allow simplification of the tests and detection of other faecal born DNA including host DNA, bacterial and viral pathogens. In an evaluation by CSIRO of the Qiagen stool kit for the direct isolation of faecal DNA, we observed false negative results in a set of 126 field samples at intolerable levels (3.17% *Haemonchus* 1.59% *Trichostrongylus*), and the correlation between PCR and visual results was not high. Because the primer sets worked well with DNA derived from purified eggs, larvae or adults we know the PCR reactions are robust. Therefore, this DNA extraction approach is not optimal. The false negative results observed are most likely due to incomplete sample disruption (egg digestion) using this commercial kit. In contrast, a direct faecal extraction procedure is now being assessed in the University of Melbourne shows very promising results and will overcome this limitation in the future.

# 4. Project objectives

1) By October 2007, to develop a DNA extraction method for nematode eggs found in sheep faeces.
2) By October 2008, to develop a quantitative real time PCR test for at least three nematode parasites of sheep. This will be specific to at a minimum the genus level.
3) By October 2008 develop a qualitative test for the presence of a number of additional key nematode parasites. These may include *Oesophagostomum columbianum*, *O. venulosum* and/or *Chabertia ovina*.
4) By October 2009, to improve the quantitative tests for the key sheep parasite species. Improvements will include:
    a. Enabling multiplexing of reactions, to increase throughput and reduce costs of diagnosis using the RT-PCR methodology.
    b. Stream-lining of the quantitation methodology to minimise the number of control reactions necessary per analysis.
5) By October 2009, to assess the feasibility of testing for drug resistance alleles in nematode populations using a faecal DNA-based PCR.

# 5. Success in achieving milestone

## 5.1. Multiplexing

A multiplex TaqMan probe assay was developed using genus-specific probes for *Haemonchus*, *Trichostrongylus* and *Teladorsagia*. Additionally a probe for the internal control, yeast, was developed. These multiplex assays have reduced the number of reactions from four to two per sample, and could potentially be reduced further to one per sample with further optimisation of an internal control. This is a considerable cost and time saving.

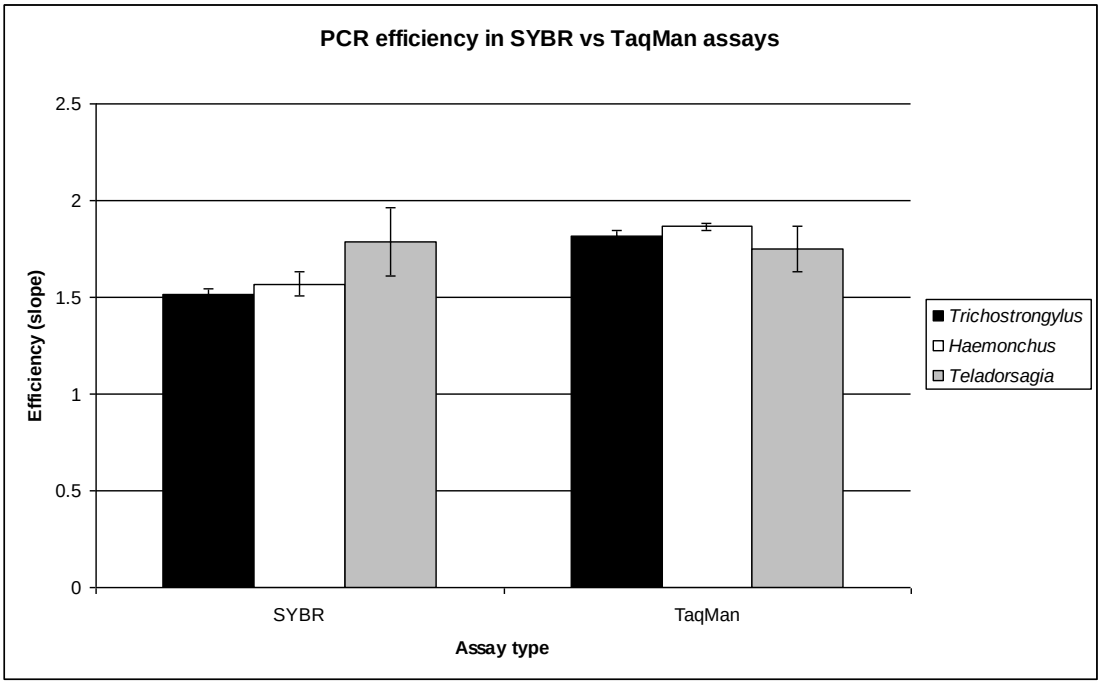
New forward primers in the ITS-2 region of the genome were designed to amplify together with the universal reverse primer NC2 (Table 1A.1), a region approximately 140 bp long. These primers all have the same five 3' bases (5'-GCAAC-3'), and are designed to amplify the same region from *H. contortus*, *Tr. colubriformis*, *Tr. vitrinus*, *Tr. axei* and *Te. circumcincta*. In the multiplex assay these primers are combined in one reaction to amplify all of these species. We have confirmed this for *H. contortus*, *Tr. colubriformis*, and *Te. circumcincta* by demonstrating that they amplify an amplicon from plasmids containing the ITS-2 region from these three nematode species.

For the internal control, yeast, primers were also redesigned to amplify an amplicon of 190 bp from the yeast ITS-2. This new reaction makes an amplicon somewhat smaller than that utilised in the SYBR green assays. The smaller amplicon sizes for both yeast and target nematodes have allowed the efficiency of the real time PCR to be increased. Figure 1A.1 and Table 1A.2 show the increase in reaction efficiency for sets of samples for the three nematode genera groups, the TaqMan assays bring the efficiencies much closer to the ideal value of 2.00. The increase in efficiency of the TaqMan method compared to the SYBR method for the *Haemonchus* and *Trichostrongylus* reactions is significant ($p=0.0077$ and $p=0.0000$ respectively).

Table 1A.1 – Primer and probe sequences used for multiplex assays

| Target | Primer sequence | Melt temp. | Probe sequence | Melt temp. |
|---|---|---|---|---|
| *Haemonchus* | Forward – TCAAGAACATATACATGCAAC Reverse – NC2 | 53°C | TET-TGACATGTATGGCGACGATGTTC-BHQ1 | 63°C |
| *Trichostrongylus* | Forward – BAGTTBAAGAATAATACATGCAAC Reverse – NC2 | 55°C | TAMRA-CCTGTATGATGTGAACGTGTTGT-BHQ2 | 62°C |
| *Teladorsagia* | Forward – GTTCAAGAATAACATATGCAAC Reverse – NC2 | 55°C | FAM-CCGTCGTAACGTTCCTGAATGAT-BHQ1 | 63°C |
| *Saccharomyces cerevisiae* (yeast) | Forward - CAGAGGTAACAAACACAAACA Reverse – TGCAATTCACATTACGTATC | 53°C | ROX-GATCTCTTGGTTCTCGCATCGAT-BHQ2 | 64°C |

Figure 1A.1 – The TaqMan assays for *Haemonchus* and *Trichostrongylus* are more efficient than the SYBR green assays




Fluorescently-labelled TaqMan probes were designed to specifically hybridize to the PCR amplicons in a genus-specific manner (Table 1A.1). TaqMan probes consist of a single stranded DNA molecule with a 'quencher' bound to the 3' end and a 'fluorophore' bound to the 5' end. When intact the probe does not emit light because the quencher is in close proximity to the fluorophore. When complementary DNA is present in the reaction with the probe, the probe binds to this. The fluorophore is subsequently separated from the probe by the exo-nuclease activity of Taq DNA polymerase, resulting in an increase in fluorescence with each cycle of the PCR. The amount of fluorescence measured by the real time PCR machine can be used to infer the amount of DNA template that was originally in the sample.

Table 1A.2 – Amplification of ITS-2 from three nematode species using 12 faecal-DNA samples, and comparing SYBR green with TaqMan assays.

| Assay Type | Species | Sample | Mean Ct | Mean PCR efficiency | Rank |
|---|---|---|---|---|---|
| **SYBR** | ***H. contortus*** | Chiswick:#222.18_11_08 | 44.05 | 1.54 | 4 |
| | | Chiswick:#234.20_10_08 | 46.67 | 1.68 | 5 |
| | | Chiswick:#245.20_10_08 | 48.41 | 2.08 | 6 |
| | | FMAHS:#50023.12_11_08 | N/A | N/A | 9 |
| | | FMAHS:#60062.12_11_08 | 30.94 | 1.37 | 2 |
| | | St Greg's:#706.19_11_08 | 33.50 | 1.53 | 3 |
| | | St Greg's:#706.3_12_08 | 29.06 | 1.73 | 1 |
| | | St Paul's:#430.12_11_08 | N/A | N/A | 9 |
| | | St Paul's:#437.25_9_08 | N/A | N/A | 9 |
| | ***Te. circumcincta*** | Chiswick:#222.18_11_08 | 39.42 | 2.15 | 7 |
| | | Chiswick:#234.20_10_08 | 38.38 | 2.04 | 6 |
| | | Chiswick:#245.20_10_08 | 34.92 | 1.92 | 4 |
| | | FMAHS:#50023.12_11_08 | 42.21 | 1.67 | 8 |
| | | FMAHS:#60062.12_11_08 | 35.47 | 1.61 | 5 |
| | | St Greg's:#706.19_11_08 | 34.26 | 1.74 | 3 |
| | | St Greg's:#706.3_12_08 | 33.34 | 1.88 | 2 |
| | | St Paul's:#430.12_11_08 | 32.78 | 1.96 | 1 |
| | | St Paul's:#437.25_9_08 | 46.17 | 1.78 | 9 |
| | ***Trichostrongylus*** | | | | |
| | | Chiswick:#222.18_11_08 | 37.87 | 1.63 | 7 |
| | | Chiswick:#234.20_10_08 | 43.38 | 1.74 | 9 |
| | | Chiswick:#245.20_10_08 | 39.95 | 1.78 | 8 |
| | | FMAHS:#50023.12_11_08 | 35.83 | 1.45 | 6 |
| | | FMAHS:#60062.12_11_08 | 32.80 | 1.47 | 2 |
| | | St Greg's:#706.19_11_08 | 33.95 | 1.51 | 3 |
| | | St Greg's:#706.3_12_08 | 30.78 | 1.55 | 1 |
| | | St Paul's:#430.12_11_08 | 34.26 | 1.57 | 4 |
| | | St Paul's:#437.25_9_08 | 35.18 | 1.43 | 5 |
| **TaqMan** | ***H. contortus*** | Chiswick:#222.18_11_08 | 37.99 | 1.91 | 5 |
| | | Chiswick:#234.20_10_08 | 39.01 | 1.81 | 6 |
| | | Chiswick:#245.20_10_08 | N/A | N/A | 9 |
| | | FMAHS:#50023.12_11_08 | N/A | N/A | 9 |
| | | FMAHS:#60062.12_11_08 | 31.98 | 1.87 | 3 |
| | | St Greg's:#706.19_11_08 | 29.08 | 1.84 | 2 |
| | | St Greg's:#706.3_12_08 | 26.09 | 1.89 | 1 |
| | | St Paul's:#430.12_11_08 | 34.93 | 1.81 | 4 |
| | | St Paul's:#437.25_9_08 | failed | N/A | N/A |
| | ***Te. circumcincta*** | Chiswick:#222.18_11_08 | N/A | N/A | 9 |
| | | Chiswick:#234.20_10_08 | N/A | N/A | 9 |
| | | Chiswick:#245.20_10_08 | failed | N/A | N/A |
| | | FMAHS:#50023.12_11_08 | failed | N/A | N/A |
| | | FMAHS:#60062.12_11_08 | 36.54 | 1.63 | 2 |
| | | St Greg's:#706.19_11_08 | N/A | N/A | 9 |
| | | St Greg's:#706.3_12_08 | N/A | N/A | 9 |
| | | St Paul's:#430.12_11_08 | 34.30 | 1.87 | 1 |
| | | St Paul's:#437.25_9_08 | N/A | N/A | 9 |

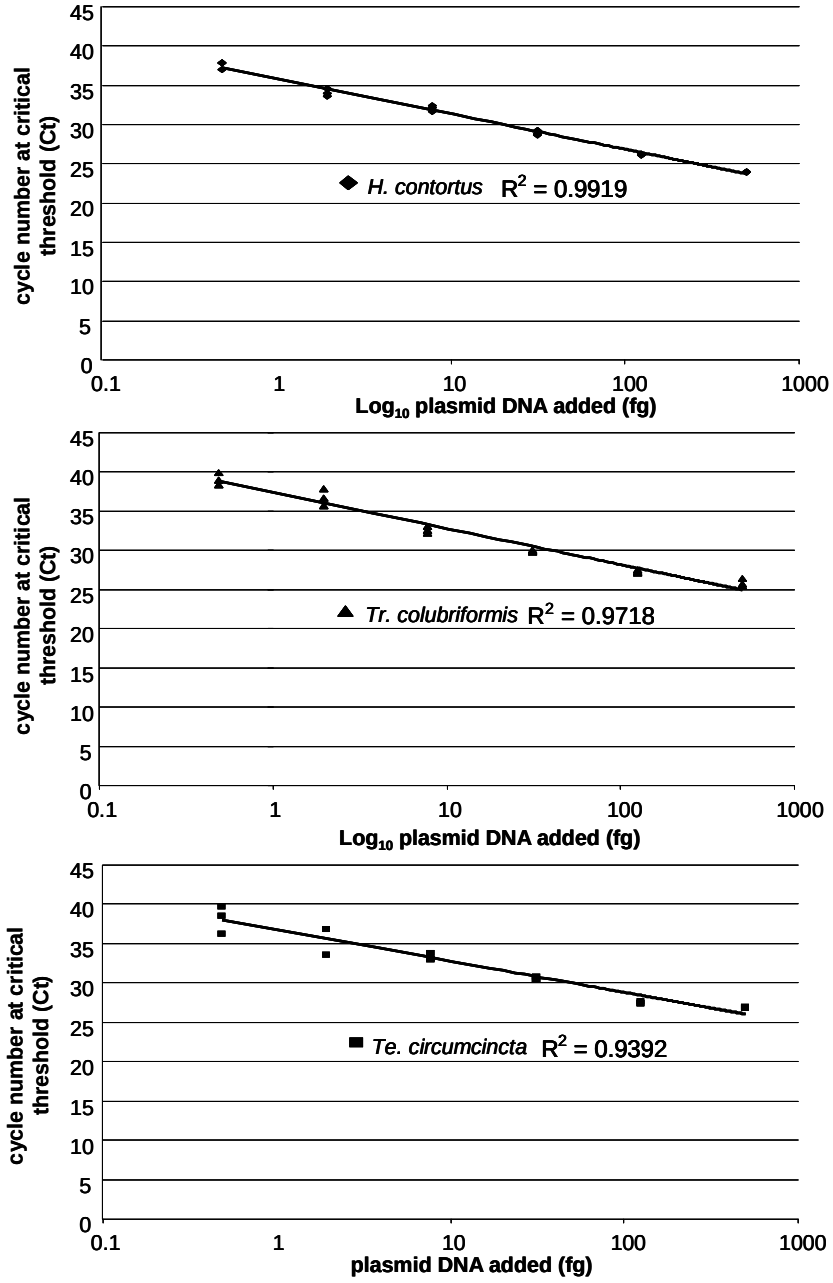
| | | | | |
|---|---|---|---|---|
| ***Trichostrongylus*** | Chiswick:#222.18_11_08 | 36.16 | 1.86 | 4 |
| | Chiswick:#234.20_10_08 | N/A | N/A | 9 |
| | Chiswick:#245.20_10_08 | N/A | N/A | 9 |
| | FMAHS:#50023.12_11_08 | 42.01 | 1.81 | 7 |
| | FMAHS:#60062.12_11_08 | 33.04 | 1.89 | 3 |
| | St Greg's:#706.19_11_08 | 29.09 | 1.85 | 1 |
| | St Greg's:#706.3_12_08 | 29.59 | 1.86 | 2 |
| | St Paul's:#430.12_11_08 | 38.26 | 1.69 | 5 |
| | St Paul's:#437.25_9_08 | 39.08 | 1.78 | 6 |

Each probe in table 1A.1 is labelled with a different coloured fluorophore allowing the simultaneous detection of multiple species. We have successfully multiplexed 3 probes within the one reaction without cross reactivity and interference. The specificity of each of the three nematode probes was confirmed by testing for cross reactivity against plasmids containing ITS2 DNA fragments from the other two major species and yeast genomic DNA. A yeast probe has similarly tested been tested against the plasmids containing the ITS2 region from the three target nematodes. The dynamic range was established for each of the 4 probes and it was found that there was a linear relationship between concentration of DNA and cycle number (Ct) from 500pg to 0.485fg of DNA, as shown in figure 1A.2.

Using a panel of field samples (see Table 1A.2), we tested the new TaqMan assays against the SYBR assays described in previous reports. As discussed above, we have been able to increase reaction efficiency by changing from SYBR to TaqMan assays. The TaqMan assays may be more susceptible to inhibition by contaminants as suggested by the number of failed reactions observed (Table 1A.2). These are preliminary findings, as there has not been sufficient time for extensive testing using the TaqMan methodology. If these results are confirmed by additional trials, it may prove necessary to include additional purification steps for faecal DNA when using TaqMan.

For the TaqMan assays that did work, the relationship with pFWEC was very good (Table 1A.3 and Figure 1A.3). For the correlation between *H. contortus* pFWEC and Ct, the TaqMan assay is comparable with that using SYBR green. For *Trichostrongylus* species, there is a substantial improvement using TaqMan compared with SYBR green. Currently, we do no yet have sufficient data to report on the correlation between *Te. circumcincta* pFWEC and Ct for the TaqMan assays.

Figure 1A.2 – The dynamic range of amplification for the TaqMan assays for *Trichostrongylus*, *Teladorsagia* and *Haemonchus*.




A multiplex approach offers the possibility of combining positive control reactions with nematode detection reactions in a single tube. We have undertaken preliminary work towards achieving this. In these experiments, it has become evident that the yeast reaction is dominating the multiplex, and nematode amplicons are made with lower efficiency and can fail when combined with the yeast reaction. We have improved the method by limiting the yeast primers to one tenth of the concentration, and doubling the concentration of the nematode specific primers. This has been only partially successful for field samples, and it is becoming evident that less yeast should be used when spiking into samples prior to DNA extraction as an additional step to avoid the problem of imbalanced reaction kinetics.

Figure 1A.3 – The relationship between calculated FWEC (pFWEC) and Ct for SYBR green compared to TaqMan assays for three groups of parasitic nematodes present in sheep faeces.

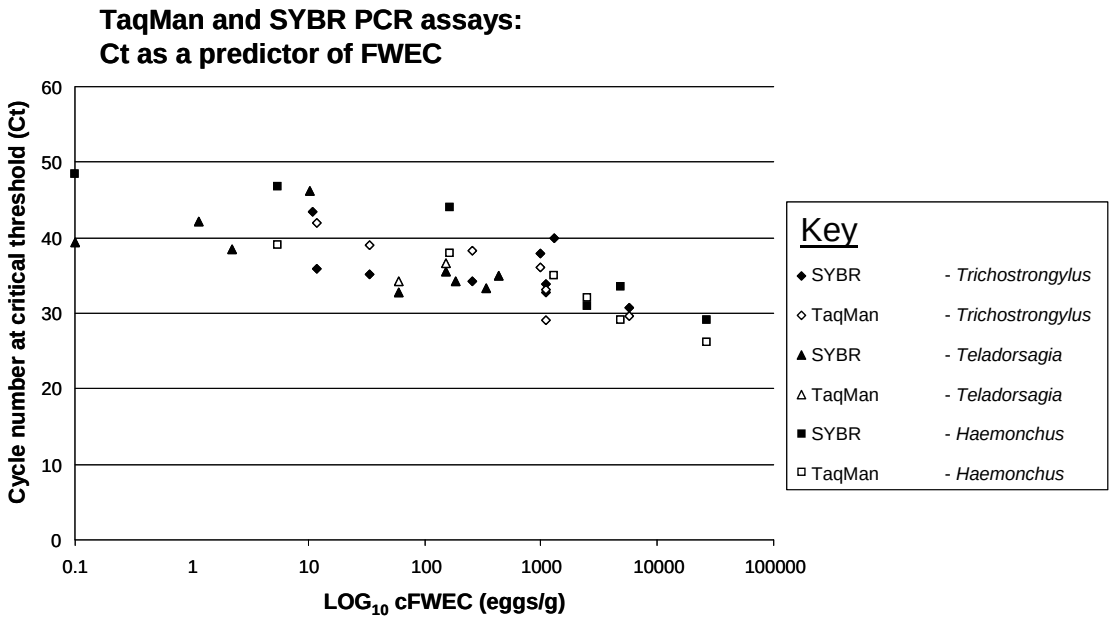



Table 1A.3 - The relationship between calculated FWEC (pFWEC) and Ct for SYBR as compared to TaqMan assays for three groups of parasitic nematodes present in sheep faeces.

| | **SYBR** | | **TaqMan** | |
|---|---|---|---|---|
| | $R^2$ (n) | pFWEC range (eggs/g) | $R^2$ (n) | pFWEC range (eggs/g) |
| ***H. contortus*** | 0.856 (6) | 0-26520 | 0.842 (6) | 5-26520 |
| ***Trichostrongylus*** | 0.261 (9) | 11-5780 | 0.792 (7) | 12-5780 |
| ***Te. circumcincta*** | 0.405 (9) | 0-433 | N/A (2) | 59-152 |

1B. Analytical methodology

In previous reports, we have shown that quantitation may be successfully achieved using two separate methodologies. First, the egg-flotation technique allows an assessment of species composition following a standard FWEC, thus allowing a simple calculation of FWEC for each species by multiplying the FWEC by the proportion of the species of interest. In a second strategy, we have created a standard curve by spiking faeces from helminth naïve sheep with nematode eggs derived from single species infections. Using this methodology we showed the quantitation of *Haemonchus*, *Teladorsagia* and *Trichostrongylus* directly from faeces-derived DNA by comparison to the standard curve. This second method was very successful, but did not translate well into some field situations where the degree of PCR inhibition was higher than for animal house samples.

Because of the limitations of the two methods described above, an attempt has been made to discover a “universal” quantitation method that does not involve standard curves or egg counts. Using samples from four sites in differing climatic zones of NSW, we assessed FWEC, the proportions of genera and genera groups present by faecal culture and larval differentiation, and also conducted PCR for *Haemonchus*, *Trichostrongylus* and *Teladorsagia*. Next we converted the FWEC data to proportional FWEC (pFWEC) by multiplying FWEC by the appropriate proportion from the larval differentiation test. For some samples, there was insufficient faecal material to conduct a culture, so for these, we calculated the mean proportions of the genera groups for the same sample date and sample site, and used these calculated proportions to calculate pFWEC.

When we assessed the relationship between pFWEC and Ct using the R-square statistic, the results indicated a reasonable relationship for *Haemonchus*, but the relationship

for *Trichostrongylus* and *Teladorsagia* was poor (Table 1B.1). To further investigate, we looked again at the R-square values, this time separating the data into sites of collection. This analysis revealed that for samples obtained from Chiswick and St Greg's college, the relationship between pFWEC and Ct is significantly better than for the samples obtained from the other two sites. This result implies that the relationship between pFWEC and Ct is site dependent and can be perturbed by, for example variations in co-purified impurities in the DNA extractions. To test for this possibility, a normalisation method utilising the Ct result for the yeast positive control was attempted. This resulted in either no change or a reduction in R-square in all cases, so was not continued. Subsequent investigation has revealed that in some cases field samples produce a low level of amplification using our yeast primers even in the absence of yeast spiking. This product has only been observed at very low levels thus far (Ct > 36), whereas the product produced when yeast is spiked into samples is typically Ct 23 to 26. The positive control therefore is a good qualitative indicator of DNA quality, but cannot be used for quantitative assessment.

Table 1B.1 – $R^2$ values are shown for the relationship between PCR results (Ct) and pFWEC (converted to species from larval differentiation)

| Name | Location | *Trichostrongylus* | *Haemonchus* | *Teladorsagia* |
|---|---|---|---|---|
| All | | 0.084 | 0.351 | 0.177 |
| Chiswick | Armidale | 0.511 | 0.368 | 0.263 |
| FMAHS | Tamworth | 0.139 | 0.329 | 0.108 |
| St Greg's | Campbelltown | 0.637 | 0.871 | 0.493 |
| St Paul's | Walla Walla | 0.157 | 0.015 | 0.162 |

An alternative use for the SYBR green assays would be for qualitative assessment of samples to indicate the presence of key genera groups. We undertook to assess the usefulness of the assays for this task.

First, the results of attempted amplification of the yeast positive control were used to screen out samples where PCR inhibition was too high to permit analysis. Of 150 samples analysed, 24 had high levels of inhibition, and amplification of the yeast product was lower than expected for at least one of three replicate reactions. From the remaining samples, the result was placed into the following four categories for each of the three genera groups assayed:

1) True – A negative Ct and a negative larval differentiation assay (LD), or a positive Ct and a positive LD.
2) neg no LD – A negative Ct where no LD was undertaken, but where the other animals in the same group which did have LD results were positive. These might be false negative results, but could also indicate that some animals in the group are not infected.
3) pos no LD - A positive Ct where no LD was undertaken, but where the other animals in the same group which did have LD results were negative. These might be false positive results, but could also indicate that the PCR has detected a low level infection which is below the limit of detection for visual methods.
4) false neg - A negative Ct where an LD was undertaken and was positive.

Table 1B.2 shows the proportions of these four categories amongst the 126 non-inhibited samples analysed for each of the three genera groups assayed.

| **Result category** | ***Haemonchus*** | ***Trichostrongylus*** | ***Teladorsagia*** |
|---|---|---|---|
| false negative | 3.17% | 1.59% | 0.00% |
| pos no LD | 25.40% | 32.54% | 51.59% |
| neg no LD | 12.70% | 3.17% | 0.00% |

| | | | |
|---|---|---|---|
| true | 58.73% | 62.70% | 48.41% |

The *Haemonchus* and *Trichostrongylus* SYBR green assays are probably not suitable for qualitative assessment in the form utilised (Hunt et al., unpublished), as the false negative result is not acceptable. Unfortunately the visual methodology currently used commercially is also sub-optimal, and this makes assessment of new tests difficult. Future work should use drench and slaughter assays as a gold standard rather than LD. LD is often not possible because of low egg counts, and we have observed much variation between microscopists in commercial laboratories when presented with identical samples. LD is clearly an unreliable technique which needs replacement with an alternative such as PCR.

Further improvements to the faecal DNA isolation procedure and the SYBR green assays are presently being undertaken in the University of Melbourne within the framework of a PhD program (Florian Roeber). Excellent results have been achieved, with no evidence of PCR inhibition and false negative results. The relationship between egg counts and Ct values remains consistent with that defined by Bott et al. (2009). The next step will be to combine the direct faecal extract procedure with the multiplex assays developed by CSIRO.

2. Detection of allelic variation

We have published evaluation of genetic variation in the *avr-14* gene from *H. contortus* in a recent paper (Hunt *et al*., 2009). Using a two stage strategy, we have shown that the different alleles we observe for this gene can be amplified from pooled DNA in such a way that the relative abundance of each allele can be evaluated.

We utilised *avr-14* primers labelled with infra-red fluorescence dyes, allowing quantitative assessment of gene, and therefore species abundance, without interfering with subsequent capillary electrophoresis used to assess allelic diversity. Figure 2.1 shows PCR results using purified *H. contortus* DNA, amplifying with ITS2 primers (multi-copy gene) and *avr-14* primers (single copy gene). Over the concentration range utilised, there is on average a 10.2 cycle difference between amplification of ITS2 and *avr-14*. This implies a 210 fold difference in copy number between the two genes, but this result could also have been affected by differing PCR efficiencies between the two sets of primers. Nevertheless, the detection of single-copy genes is clearly a greater challenge than for multiple copy genes such as the ribosomal RNA genes.

Figure 2.2 shows the detection of multiple alleles from some of the same PCR reactions illustrated in Figure 2.1. An overlay graph is represented in the figure illustrating the repeatability of this type of test. The PCRs all had differing concentrations of starting DNA, indicating that the starting concentration does not affect the relative size of the peaks. Comparison to the inset shows that the trace from the capillary electrophoresis matches somewhat with frequency histogram data assembled from many PCRs from individual worms. This has been shown previously (Redman *et al*., 2008), and the method suggests that assaying allelic variation in this way may be a cost effective solution for field situations.

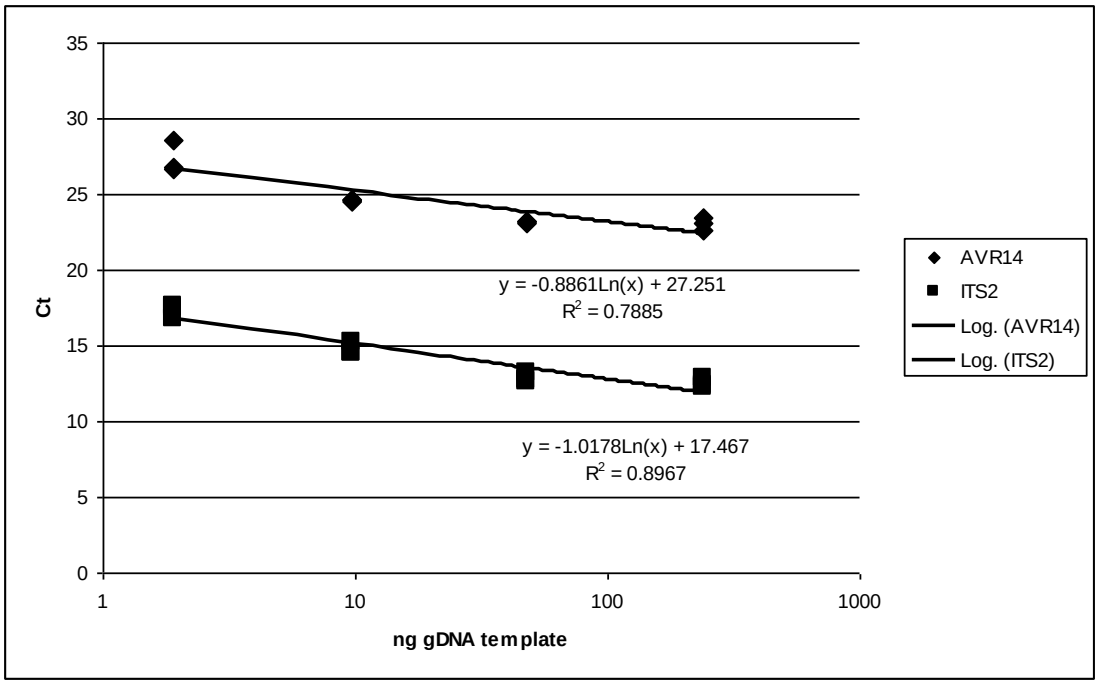



Figure 2.1 – The graph shows qPCR amplification of *avr-14* (diamonds) and ITS-2 (squares) as assessed by critical threshold cycle (Ct – y-axis). Four different concentrations of purified *H. contortus* DNA were used (see x-axis). The DNA was purified from >1,000 ethanol-dried adult worms and a mixture of the Wallangra2003 and ChiswickSM1977 isolates were used.

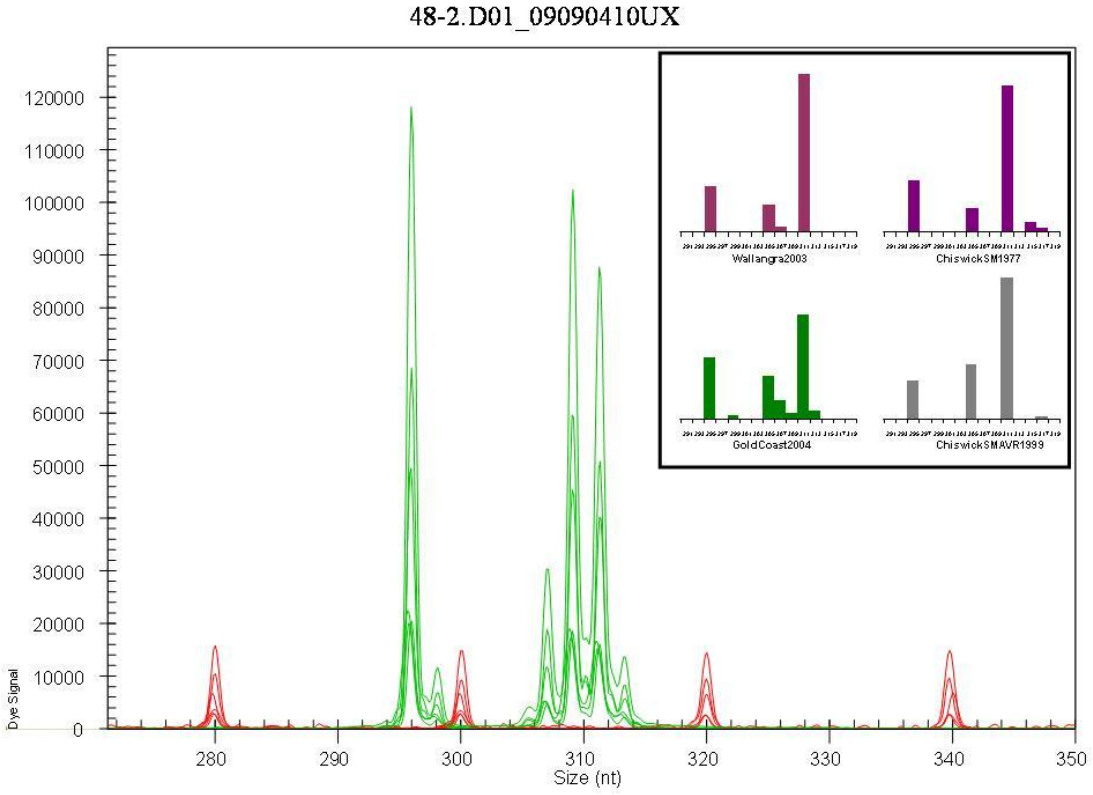



Figure 2.2 – The graph shows the results of capillary electrophoresis following qPCR detection. Samples from figure 2.1 were loaded onto the Beckman Coulter 8000 capillary electrophoresis machine, and fragments (green) run in conjunction with size standards (red).

The figure shows an overlay of six samples, illustrating the reproducibility of results. The similarity to the allele frequency histogram for a number of isolates (inset) can be seen. The y-axis shows relative fluorescence and the x-axis the calculated size of the fragments in base pairs (calculated from the size standard).

# 6. References

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E. Redman, E. Packard, V. Grillo, J. Smith, F. Jackson, and J. S. Gilleard. (2008) "Microsatellite Analysis Reveals Marked Genetic Differentiation Between *Haemonchus contortus* Laboratory Isolates and Provides a Rapid System of Genetic Fingerprinting. *International Journal for Parasitology* **38**: p111-122.

# 7. Recommendations

Milestone 6 was readily achieved. Based on the progress, there is considerable promise for the real-time PCR approach to be applied as diagnostic and epidemiological/ecological tools. Further work is required however before commercial interests could take on these tests as a routine procedure.